# CHARACTERIZATION OF GROUP B STREPTOCOCCAL SURFACE ANTIGENS DELTA, EPSILON, AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

By

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by

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To my mother and father.

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TABLE OF CONTENTS

page

ACKNOWLEDGMENTS ..... iv

LIST OF TABLES ..... viii

LIST OF FIGURES ..... ix

KEY TO ABBREVIATIONS ..... xi

ABSTRACT ..... xiii

CHAPTER

1 INTRODUCTION ..... 1

Group B Streptococcal Disease ..... 1
Group and Type Specific Antigens of Group B Streptococci ..... 3
Other Surface Antigens of Group B Streptococci ..... 5
Pathogenesis of Group B Streptococci ..... 9
Avoidance of Immune Clearance ..... 13
Vaccine Development ..... 15
Specific Aims ..... 19

2 ISOLATION AND CHARACTERIZATION OF THE δ AND ε ANTIGENS ..... 24

Introduction ..... 24
Materials and Methods ..... 26
Results ..... 34
Discussion ..... 40

3 EVALUATION OF GENETIC COMPONENTS INVOLVED IN δ AND ε EXPRESSION ..... 61

Introduction ..... 61
Materials and Methods ..... 63
Results ..... 70
Discussion ..... 76

4 THE ASSOCIATION OF δ AND ε EXPRESSION WITH PATHOGENESIS OF SEROTYPE III GBS ....93

Introduction....93
Materials and Methods ....94
Results....96
Discussion....97

5 CHARACTERIZATION OF GROUP B STREPTOCOCCAL GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE: SURFACE LOCALIZATION, ENZYMATIC ACTIVITY, AND PROTEIN-PROTEIN INTERACTIONS ....102

Introduction....102
Materials and Methods ....104
Results....111
Discussion....114

6 SUMMARY AND CONCLUSIONS ....125

Isolation and Characterization of the δ and ε Antigens ....126
Determination of Genetic Components Involved in δ and ε Expression....131
The Association of δ and ε Expression in the Pathogenesis of Serotype III GBS ...136
Characterization of Group B Streptococcal Glyceraldehyde-3-Phosphate Dehydrogenase: Surface Localization, Enzymatic Activity, and Protein-Protein Interactions....138
Conclusions....139

LIST OF REFERENCES ....142

BIOGRAPHICAL SKETCH ....163

LIST OF TABLES

Table page

1-1 Expression of δ/ε antigens on serotype III group B streptococci isolated from septic vs. colonized sources. ....22

1-2 In vitro opsonophagocytic killing of group B streptococci by human peripheral blood leukocytes. ....23

3-1. Primers used in this study. ....83

3-2. Epsilon expression by group B streptococci (GBS) of RDP types 1-4. ....89

4-1. Results of passive protection assays ....101

LIST OF FIGURES

Figure page

1-1. Identification of ε reactivity on serotype III, δ-positive group B streptococci by radioimmunoassay ....20

1-2. Evaluation of anti-δε, anti-δ, and anti-ε reagents with group B streptococcal (GBS) whole cells by ELISA....21

2-1. Detection of δ/ε in sonic extracts of group B streptococcal (GBS) whole cells by competition ELISA....48

2-2. Anion-exchange chromatography of GBS sonic extracts. ....49

2-3. Detection of δ and ε in anion-exchange column fractions by competition ELISA..50

2-4. Evaluation of proteinase K sensitivity of the δ antigen on A909 (serotype Ic/αβγδ) whole cells by ELISA....51

2-5. Evaluation of sodium metaperiodate sensitivity of the δ antigen on A909 (serotype Ic/αβγδ) whole cells by ELISA....52

2-6. Evaluation of anti-δε antiserum reactivity with material contained in anion-exchange column fractions from DL700 (serotype III) and J48 (serotype III/δε) ...53

2-7. Evaluation of anti-δε antiserum reactivity with sodium-dodecyl sulfate (SDS)-extracted material from representative δε-surface positive and δε-surface negative GBS strains....54

2-8. Visualization of ε by Western immunoblot....55

2-9. Evaluation of proteinase K sensitivity of the ε antigen on J48 (serotype III/δε) whole cells by ELISA ....56

2-10. Evaluation of sodium metaperiodate sensitivity of the ε antigen on J48 (serotype III/δε) whole cells by ELISA ....57

2-11. Evaluation of sodium metaperiodate sensitivity of the ε antigen assessed by Western immunoblot ....58

2-12. Western immunoblot analysis of high-molecular weight material gel-purified from sonic extracts of DL700 (lane 1) and J48 (lane 2) ....59

2-13. Western immunoblot analysis of SDS-extracted material from group B streptococcal (GBS) whole cells. ....60

3-1. Western immunoblot analysis of recombinant GBS acyl-carrier protein ....84

3-2. Polymerase Chain Reaction (PCR) amplification of *srr* DNA from GBS strains. ..85

3-3. Detection of *srr* by Southern hybridization....86

3-4. Ribonucleic Acid (RNA) dot blot to detect *srr* mRNA....87

3-5. Comparison between J48 (serotype III/δε) and DL700 (serotype III) of ORFs contained within an operon including the *srr* variant common to δε-negative GBS strains. ....88

3-6. Detection of a variant *srr* ORF associated with serotype III/δε GBS by Southern hybridization....90

3-7. Western immunoblot analysis of recombinant Srr from J48 (serotype III/δε) ....91

3-8. Comparisons of *srr*-containing loci representative of non-RDP III-3 and RDP III-3 GBS ....92

4-1. Comparison of $LD_{90}$ values of serotype III δε-positive and δε-negative strains ...100

5-1. Protein stain and Western immunoblot analysis of GBS GAPDH. ....119

5-2. Detection of surface-localized GBS GAPDH by ELISA using an anti-Plr monoclonal antibody ....120

5-3. Evaluation of GAPDH enzymatic activity associated with GBS whole cells....121

5-4. Evaluation of GBS *gapC* copy number by Southern hybridization. ....122

5-5. Western immunoblot analysis to detect binding of extracellular matrix and cytoskeletal proteins to immobilized GBS GAPDH. ....123

5-6. ELISA to detect binding of GBS GAPDH to immobilized extracellular matrix and cytoskeletal proteins ....124

## KEY TO ABBREVIATIONS

| | |
|---|---|
| cpm | counts per minute |
| Ci | Curie |
| CPS | capsular polysaccharide |
| CsCl | cesium chloride |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediamine-tetraacetate |
| ELISA | enzyme linked-immunosorbent assay |
| g | gravity |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GAS | group A streptococci |
| GBS | group B streptococci |
| h | hours |
| IgA | immunoglobulin A |
| IgG | immunoglobulin G |
| kDa | kilodaltons |
| kb | kilobase |
| min | minutes |
| $M_r$ | relative molecular weight |
| NADH | nicotinamide adenine dinucleotide, reduced form |
| nm | nanometer |
| nt | nucleotide |

| | |
|---|---|
| OPD | o-phenylene-diamine |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | 0.15 M phosphate buffered saline, pH 7.4 |
| PCR | polymerase chain reaction |
| s | seconds |
| Tris | tris(hydroxymethyl)aminomethane |
| TT | tetanus toxoid |
| SDS | sodium dodecyl sulfate |

Abstract of Dissertation Presented to the Graduate School
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PARTIAL CHARACTERIZATION OF GROUP B STREPTOCOCCAL SURFACE ANTIGENS DELTA, EPSILON, AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

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Group B streptococci (GBS) are an important cause of disease in both neonates and immunocompromised adults. Of the nine serotypes identified, serotype III GBS were found in over 90% of isolates from cases of meningitis. Previous studies show, that in addition to the group and serotype polysaccharides, proteins are also found at the cell surface. Two novel antigenic reactivities, δ and ε, are co-expressed on a subset of serotype III GBS, and are present more frequently on isolates from septic vs. colonized sources. This study demonstrates preliminary characterization of the δ and ε antigens. The δ antigen is destroyed by proteolysis and is associated with one of the three known GBS acyl-carrier proteins. The ε antigen is a high molecular weight glycoprotein with similarity to serine rich repeat (Srr) proteins such as GspB and Hsa of *Streptococcus gordonii* and Fap1 of *Streptococcus parasanguis*. Restriction digest pattern (RDP) typing has identified a more pathogenic subset of serotype III GBS, designated RDP III-3. All

RDP III-3 (but not RDP III-1, 2, or 4) isolates express the ε antigen. The *srr* gene from RDP III-3, δε-positive GBS differs from *srr* from all non-III-3 strains. Anti-ε antiserum binds to the recombinant Srr from a RDP III-3 strain, suggesting that the Srr protein is the antigenic backbone of the ε glycoprotein. The *srr* gene in both III-3 and non-III-3 strains are contained within loci including accessory *secA* and *secY* homologues. The organization of the *srr*-containing loci is different for RDP III-3 and non-III-3 GBS strains. In a mouse model of neonatal sepsis, serotype III/δε-positive strains demonstrated $LD_{90}$ values 2-4 logs lower than serotype III/δε-negative strains. Results of this study indicate both antigenic and genetic differences that distinguish a more pathogenic subset of serotype III GBS and may lead to improved diagnostic capabilities or improved vaccine candidates in an effort to reduce the incidence of GBS disease.

# CHAPTER 1
# INTRODUCTION

## Group B Streptococcal Disease

Lancefield group B streptococci (GBS) are a major cause of neonatal sepsis (Baker and Edwards 1994) and are important pathogens in immunocompromised adults (Farley 2001). There are approximately 19,000 cases of invasive GBS disease per year in the United States, resulting in 1,900 deaths (CDC 2002). There are two distinct syndromes of neonatal GBS disease: early- and late-onset. Early-onset disease occurs within the first week of life and is vertically transmitted from mother to infant. It is assumed that the main route of infection is aspiration of GBS during parturition, resulting in subsequent colonization of the respiratory epithelium (Baker and Edwards 1994). All known GBS serotypes have been isolated from infants suffering from early-onset disease; however, serotypes Ia, Ib, II, III, and V are more commonly isolated in the United States (Harrison et al. 1998). Three manifestations of early-onset disease include sepsis with no known focus of infection, meningitis, and pneumonia. Before the Centers for Disease Control and Prevention (CDC) established guidelines for the treatment and prevention of GBS infections in 1996 (CDC 1996), 80% of all GBS infections were early-onset (Baker and Edwards 1994). Intrapartum prophylaxis has reduced the percentage of early-onset disease to 50% of total GBS disease (CDC 1997).

Important risk factors for early-onset disease include maternal carriage of GBS (Boyer et al. 1983), having a previous infant with GBS disease, having a heavily colonized mother (Gerards et al. 1985, Lim et al. 1982), or having a mother with a low

level of antibodies to the capsular polysaccharide (Baker et al. 1977). There are also a number of obstetric risk factors, which include premature rupture of membranes (Schuchat et al. 1994), prolonged labor (Schuchat et al. 1994), intrapartum fever (Boyer et al. 1983, Schuchat et al. 1994), and urinary tract infection during pregnancy (Liston et al. 1979, Schuchat et al. 1994).

Late-onset disease occurs from 1 week to 3 months after birth. Serotype III GBS were responsible for approximately 90% of all late-onset infections (Baker and Edwards 1994), with meningitis being a common clinical manifestation that results in a substantial number of survivors suffering permanent neurological damage. The acquisition of GBS resulting in late-onset disease is less well understood. It is speculated that the organism can be acquired during passage through the birth canal similar to early-onset disease, as 50% of GBS isolated from late-onset infections are the same serotype as the colonized mother (Anthony et al. 1979, Dillon et al. 1987). Nosocomial and community sources are also believed to contribute to late-onset infections (Trager et al. 1996). Intrapartum prophylaxis has been successful in reducing the incidence of early-onset disease, but the incidence of late-onset disease has remained unchanged (Schrag et al. 2000).

Although invasive GBS disease occurs in healthy adults, most disease in non-pregnant adults occurs in those with underlying medical conditions such as diabetes, alcoholism, and cancer (Farley et al. 1993, Farley 2001, Schuchat 1998). Clinical manifestations are numerous and varied, and the mode of transmission is unknown. Skin and soft tissue infections are the most frequently reported syndromes (Farley 2001). Infected foot ulcers are common in patients with diabetes. Breast cellulitus has also been reported in instances of breast conservation therapy for stage I or II breast cancer (Mertz

et al. 1998). Up to 23% of invasive GBS disease in adults presents as a urinary tract infection (Farley et al. 1993, Munoz et al. 1997, Schwartz et al. 1991, Trivalle et al. 1998, Tyrrell et al. 1996, Verghese et al. 1986). Meningitis can occur, but is not common in adults (Farley et al. 1993). Other manifestations also include pneumonia, endocarditis (Bayer et al. 1976, Farley et al. 1991, Gallagher and Watanakunakorn 1985, Lerner et al. 1977), bone and joint infections (Garcia-Lechuz et al. 1999), and pyogenic arthritis (Nolla et al. 2003). The most common serotypes associated with adult disease are Ia, III, and V, in approximately equal proportion with minor geographic variations (Tyrrell et al. 2000).

GBS are sensitive to treatment with penicillin G and ampicillin (Baker et al. 1981, Bayer et al. 1976). Clindamycin and erythromycin are also used clinically, although resistance to these antibiotics is increasing (Pearlman et al. 1998). Aminoglycosides have little activity when used alone, but may provide a synergistic effect when combined with ampicillin or penicillin G (Baker et al. 1981).

**Group and Type Specific Antigens of Group B Streptococci**

Lancefield (1933, 1934, 1938) devised a system of categorizing streptococci based on hot-acid extracted surface polysaccharides, called group antigens. The group B carbohydrate of GBS is comprised of four sugars: rhamnose, N-acetylglucosamine, galactose, and glucitol (Carey et al. 1980, Heidelberger et al. 1967, Kane and Karakawa 1977, Michon et al. 1987, 1988, Pritchard et al. 1981,1984), in a complex, highly branched arrangement (Michon et al. 1991). It has been known for some time that the group B polysaccharide is in close proximity to the cell wall (Wagner et al. 1980) and it was recently suggested that the group B antigen is covalently linked to N-acetylmuramic acid residues of the disaccharide repeating unit of cell wall peptidoglycan (Deng et al.

2000). Expression of the group B carbohydrate is not regulated by growth rate (Ross et al. 1999). In mice, antibodies against the group B carbohydrate are not protective against lethal challenge of GBS (Lancefield et al. 1975). Antibodies to the group B carbohydrate do not appear to contribute to protective immunity against GBS in humans either (Anthony et al. 1985).

With few exceptions, all GBS strains are encapsulated. Four carbohydrates (glucose, galactose, N-acetylglucosamine, and sialic acid) comprise the GBS polysaccharide capsule with the exception of serotypes VI and VIII, which lack N-acetylglucosamine (Kogan et al. 1996, von Hunolstein et al. 1993). GBS were subdivided into serotypes based on the linkages of the sugars that comprise the capsule. To date, nine serotypes have been identified: Ia, Ib, II, III, IV, V, VI, VII, and VIII; and the structures of the capsular polysaccaharides of all have been determined (DiFabio et al. 1989, Hunolstein et al. 1993, Jennings et al. 1983a, 1983b, Kogan et al. 1994, 1995, 1996, Wessels et al. 1987, 1991).

Investigators have suggested that the capsular polysaccharide is linked to peptidoglycan (DeCueninck et al. 1982, Yeung and Mattingly 1986). Analysis of digested insoluble cell-wall fragments from lysates of a serotype III GBS suggests that serotype polysaccharide is linked via a phosphodiester bond and an oligosaccharide linker to the N-acetylglucosamine repeating unit of peptidoglycan, and its linkage to peptidoglycan is independent of the group B carbohydrate (Deng et al. 2000).

The capsular polysaccharide appears to be a major factor associated with virulence of GBS. Acapsular and asialo mutants are more susceptible to opsonophagocytosis in the presence of complement and PMNs than their encapsulated parent strains (Marques et al.

1992, Rubens et al. 1987, Wessels et al. 1989). The serotype III capsular polysaccharide is one of the three major capsular types associated with invasive neonatal infection and the most common serotype isolated from cases of meningitis (Dillon et al. 1987, Wenger et al. 1990). The importance of the terminal sialic acid residue of the serotype III polysaccharide has been documented. An asialo GBS mutant was less virulent than its fully encapsulated parent in opsonophagocytosis assays (Wessels et al. 1989). Sialic acid was also shown to contribute to complement activation and generation of a specific antibody response (Markham et al. 1982).

**Other Surface Antigens of Group B Streptococci**

In addition to the group B carbohydrate and serotype-specific polysaccharides, numerous protein antigens can be associated with the GBS cell surface. Several surface proteins from group B streptococci have been identified in an attempt to characterize the complex nature of the cell wall and polysaccharide capsule. Wilkinson and Eagon (1971) first described the c-protein in 1971 using a polyclonal rabbit antiserum made against whole formalin-killed cells of CDC strain A909 (serotype Ia). Antibodies against the group- and serotype-specific carbohydrates in the polyclonal rabbit antiserum were eliminated by exhaustive adsorption with a c-protein-negative serotype Ia strain, and the non-serotype Ia antigens that the antiserum bound were called the c-protein. It was later recognized that c-protein typing serum actually recognizes at least four antigenic moieties: α, β, (Bevanger 1985, Johnson and Ferrerri 1984, Wilkinson and Eagon 1971) γ, and δ (Brady et al. 1988, Chun 1991). The genes encoding the α and β proteins have been cloned, (Cleat and Timms 1987, Michel et al. 1991), and the proteins characterized.

The α protein is a large, highly repetitive protein resistant to cleavage by trypsin (Wilkinson and Eagon 1971). This protein contains nine long tandem repeats of 82 amino acids each. These repeats constitute >75% of the total size of the protein, and the protein itself has a typical wall-anchoring LPXTG motif (Michel et al. 1992). Deletion of tandem repeats is associated with escape from protective immunity, increased pathogenicity, and vertical transmission in mice (Gravekamp et al. 1998, Madoff et al. 1992, 1996). The β antigen has been characterized as a human IgA-binding protein (Russel-Jones et al. 1984). The γ reactivity has been found to correspond to a variant amino-terminus of the α protein (Madoff, personal communication). The nature of the δ antigen has been partially determined and is described below. In addition to antigens reactive with c-protein typing antiserum, additional GBS protein antigens include protein Rib (Stalhammar-Carlemalm et al. 1993), the R and X proteins (Ferrieri 1988), C5a peptidase (Beckmann et al. 2002, Cleary et al. 1992), surface immunogenic protein (Sip) (Brodeur et al. 2000), laminin-binding protein (Lmb) (Spellerberg et al. 1999), Fbs, and a Rib-like protein (Areschoug et al. 1999).

The α, β, and γ antigens are expressed primarily by serotype Ia, Ib, and II organisms, while δ and Rib have been reported to be expressed by most serotype III strains (Brady et al. 1988, Chun, 1991, Stalhammar-Carlemalm et al. 1993). Because of the higher association of δ antigen expression with serotype III GBS isolates from septic vs. colonizing sources (Brady et al. 1996), a polyclonal rabbit antiserum against J48 (serotype III/δε) whole cells was generated to facilitate further study of this marker. The anti-J48 antiserum was rendered more specific for the previously described δ antigen by adsorption with GBS strain DL700 (serotype III) whole cells to remove antibodies

against the serotype III polysaccharide and group B carbohydrate, as well as any other common surface determinants. The adsorbed polyclonal rabbit antiserum (Brady et al. 1988) was subsequently absorbed with GBS strain A909 (serotype Ic/αβγδ) to remove antibodies against δ. The DL700- and DL700/A909-adsorbed reagents were then used to evaluate reactivity of a panel of known δ-positive and δ-negative GBS strains by radioimmunoassay (Figure 1-1). The DL700-adsorbed reagent reacted with A909 (serotype Ic/αβγδ) and serotype III, δ-positive strains; whereas the DL700/A909-adsorbed reagent antiserum was not reactive with strain A909 but retained reactivity with serotype III, δ-positive strains. These results indicated that another immunological reactivity absent from strain A909 (serotype Ic/αβγδ) was associated with the δ antigen on serotype III strains. The newly identified antigen was designated ε. Thirty serotype III/δ strains tested all expressed ε. None expressed ε without δ, and vice versa. Polyclonal reagents reactive with both δ and ε antigens (anti-δε), or with each of antigen individually (anti-δ or anti-ε) can be generated by selective adsorption of polyclonal rabbit antisera as described (Brady et al. 1988, 1996). For the work described below, a δ-reactive reagent was generated by adsorption of anti-c protein typing antiserum with strain ss618 (serotype Ib/αβγ) to remove anti-α, β, and γ antibodies. A δε-reactive reagent was generated by adsorption of an anti-J48 whole cell antiserum with strain DL700, and an ε-reactive reagent was generated by adsorption of the anti-J48 antiserum with strain DL700 and A909. The reactivity of these reagents with strains of GBS including those used for immunizations and adsorptions is shown in Figure 1-2.

In addition to the polysaccharide capsule, serotype III GBS isolates can be further classified into four distinct lineages based on *Hind*III and *Sse*83871 restriction digest

patterns (RDPs) of chromosomal. These have been designated RDP types III-1, 2, 3, and 4 (Takahashi et al. 1998). Most (91%) serotype III invasive neonatal isolates have been reported to be RDP III-3, suggesting that these strains may represent a more virulent lineage of serotype III GBS.

Other methods for subtyping GBS have also been reported and include multilocus enzyme electrophoresis (MLEE) (Quentin et al. 1995), ribotyping (Chatellier et al. 1996), randomly amplified polymorphic DNA (RAPD) (Chatellier et al. 1997), and most recently by pulsed-field gel electrophoresis of *Sma*I-digested chromosomal DNA (Rolland et al. 1999). None of these methods subdivide GBS strains into pathogenic/nonpathogenic groups.

Pulsed-field gel electrophoresis of 10 mother/child isolate pairs (Melchers et al. 2003) supports the findings of other investigators (Chatellier et al. 1996, 1997, Quentin et al. 1995, Rolland et al. 1999) that the heterogeneity within GBS isolates makes it difficult to definitively identify virulent strains based on entire chromosomal DNA. Capsular polysaccharide genes (Kong et al. 2002a), surface protein genes (Kong et al. 2002b), and mobile genetic elements (Kong et al. 2003) have been used in combination to characterize 224 GBS isolates. Such a combinatorial approach identified 56 different genotypes and eight genetic clusters, again illustrating the heterogeneity of GBS isolates. A multilocus sequence typing system (MLST) using seven housekeeping genes has also been developed, and has identified a sequence type (ST-17) that correlates with neonatal invasive disease, much like that of RDP III-3 strains (Jones et al. 2003).

The RDP III-3 data reported by Takahashi et al. (1998) showed that while 33% of vaginal isolates were III-3, 91% of invasive neonatal isolates were III-3. These data were

reminiscent of an epidemiological study by Brady et al. (1996), in which δ/ε co-expression was detected on 83.3% of serotype III GBS from septic sources, compared to 58.8% of serotype III isolates from colonized sources (Table 1-1). Taken together, these results suggested that the δ/ε antigenic markers and RDP III-3 genetic marker might identify the same highly pathogenic lineage of serotype III GBS.

## Pathogenesis of Group B Streptococci

GBS are associated with asymptomatic colonization in healthy adults. However, in susceptible individuals, GBS can be a devastating pathogen. Pathogenesis is multi-factoral, and is generally considered to follow a progression that begins with adhesion to host epithelial cells, invasion of tissue, direct injury to host tissue, and finally induction of sepsis (Fischetti et al. 2000). In addition to affecting the host directly, a major component of GBS pathogenesis is avoiding host immune mechanisms.

The initial step in GBS disease of neonates is asympomatic colonization of the female urogenital tract. As expected, GBS bind efficiently to human vaginal cells (Zawaneh et al. 1979). GBS can also adhere to placental membranes, alveolar epithelium and endothelium, and pharyngeal mucosa, which could be relevant to vertical transmission and induction of disease in a newborn infant (Fischetti et al. 2000). The mechanism of adherence to host tissues has not been completely elucidated. However a number of potential adhesins and GBS factors have been shown to affect GBS binding to host cells (Tamura et al. 1994, Wibawan et al. 1992).

After initial adherence, GBS must penetrate and survive inside different host tissues. GBS have been shown to migrate through freshly isolated chorioamniotic membranes (Galask et al. 1984), as well as isolated chorion cells from human

cesarean-section placentas (Winram et al. 1998). Invasion of these cell types was necessary to cause amniocentesis and exposure of the fetus to GBS. Interestingly, although able to invade chorion cells, GBS were unable to invade amnion cells under a number of assay conditions (Winram et al. 1998), suggesting that the amnion may provide a formidable host barrier to infection.

In most instances, GBS must traverse the alveolar epithelium, the pulmonary interstitium, and the pulmonary endothelium to gain access to the systemic circulation and cause bacteremia and sepsis after aspiration of infected amniotic fluid. Studies using a primate model of infection have shown GBS within alveolar epithelial cells, interstitial fibroblasts, and capillary endothelial cells (Gibson et al. 1993, Rubens et al. 1991). Tissue-culture studies have also shown invasion of human lung epithelial cells (Rubens et al. 1992), as well as human umbilical vein and piglet endothelial cells (Gibson et al. 1993). Quantitative tissue-culture studies have shown that GBS isolates of serotypes Ia, Ib, Ic, II, and III are capable of invading alveolar epithelial cells with a small degree of strain-to-strain variation (Hulse et al. 1993, Rubens et al. 1992). Interestingly, the capsular polysaccharide appears not to be necessary for invasion of respiratory epithelial cells (Hulse et al. 1993) and attenuates invasion of umbilical vein endothelial cells (Gibson et al. 1993) and alveolar epithelium (Tamura et al. 1994), suggesting that factors other than the capsular polysaccharide are responsible for invasion of host tissue. In vivo and in vitro studies have shown that GBS were able to invade and transverse the host cells necessary to gain access to the systemic circulation of an infected host.

After gaining access to the circulatory system of the host, GBS must bind and invade brain microvascular endothelial cells, which constitute the blood-brain barrier, in

order to cause meningitis. GBS have been shown to invade brain microvascular cells in tissue culture (Nizet et al. 1997). Serotype III GBS were better at invading brain microvascular cells than other serotypes, suggesting this as at least one reason serotype III cause >90% of GBS meningitis cases. The same study suggested that GBS were able to directly injure the blood-brain barrier, although the exact mechanism of such damage is not fully understood.

Direct injury of host tissue by GBS appears to contribute to pathogenesis. One of the clinical presentations of early-onset disease is severe pneumonia. GBS was shown to cause extensive pulmonary damage in pneumonia (Vollman et al. 1976). A hemolysin of GBS has cytopathic effects on pulmonary epithelial cells (Gibson et al. 1999, Nizet et al. 1996), suggesting that the hemolysin of GBS contributes to pneumonia. Degradation of host-cell hyaluronic acid by hyaluronate lyase (Lin et al. 1994) may also play a role in GBS pathogenesis, although the exact biological role is uncertain. GBS were able to degrade collagen (Jackson et al. 1994), which may be important for penetration of the chorioamnion. Lipoteichoic acid (LTA) has been shown to be cytotoxic for a variety of human cell monolayers in tissue culture, including human embryonic brain cells and human embryonic amnion cells (Miyazaki et al. 1988), although LTAs role in vivo is uncertain. It has also been suggested that unidentified proteases are able to degrade placental tissue (Schoonmaker et al. 1989).

If GBS are able to avoid host immune clearance mechanisms and establish bacteremia, sepsis may be the consequence. Sepsis is characterized by extensive hemodynamic alterations leading to a decreased cardiac output, acidosis, and finally, mutiple organ failure. Recent work using animal models of neonatal infection has

improved the understanding of how GBS activate host inflammatory mediators and contribute to sepsis and circulatory shock. In animals, intravenous injection of GBS leads to acute (<1 h) increases in pulmonary artery pressure and decreased arterial oxygenation (Gibson et al. 1992). Pulmonary hypertension and hypoxemia persist through the late phase of sepsis (2-4 h). A role of the inflammatory mediator TNF-α is suggested but not established. In septic individuals, there is increased TNF-α in the bloodstream, and is associated with edema, neutropenia, collapse of blood vessels, and eventually, multiple organ failure. TNF-α can be detected in the blood, urine, and cerebral spinal fluid in infants with invasive disease (Williams et al. 1993). However, anti-TNF-α antibodies do not affect GBS mortality in mice or rats (Teti et al. 1992, 1993). Cell wall preparations of GBS cause TNF-α release from human monocytes (Medvedev et al. 1998). The group B carbohydrate and peptidoglycan have both been reported to be better than either LTA or capsular polysaccharide at stimulating TNF-α release from monocytes (Vallejo et al. 1996). Serotype III capsular polysaccharide was reported to have no effect on TNF-α production by human mononuclear cells in vitro (Williams et al. 1993). Activation of nitrous oxide by GBS hemolysin has been reported to contribute to sepsis and hypotensive shock (Griffiths and Rhee 1992, Ring et al 1998). The hemolysin of GBS has recently been identified within a cluster of at least 10 open reading frames in a single operon (Spellerberg et al. 1999).

Antibodies and complement are both necessary for immune clearance of GBS. Antibodies directed against the capsular polysaccharide, but not the group B carbohydrate, have been shown to be protective in a neonatal mouse model of infection (Lancefield 1934, 1938). Numerous studies have been conducted in an attempt to identify

antigens in addition to the capsular polysaccharide capable of eliciting opsonic antibodies. Lancefield et al. (1975) showed protection by rabbit antiserum against unidentified "c-protein" antigens independent of the polysaccharide capsule of GBS. Madoff et al. (1991) showed that monoclonal antibodies recognizing the α antigen are protective in mice. It has since been shown that antibodies to both the amino-terminal domain and the repeat region of the α protein are protective in an animal model of GBS infection and mediate opsonophagocytic killing in vitro (Kling et al. 1997). Antibodies against the β antigen are also protective in mice (Fusco et al. 1997, Madoff et al. 1992, Michel et al. 1991). Antibodies to protein Rib confer protective immunity (Stalhammar-Carlemalm et al. 1993), as does antiserum recognizing an α-like protein present on serotype V GBS (Lachenauer and Madoff 1996). These studies suggest that antibodies to surface structures in addition to the capsular polysaccharide are important targets of protective immunity.

**Avoidance of Immune Clearance**

GBS have many ways to resist opsonophagocytosis and survive inside macrophages. For example, in serotype III GBS, resistance to opsonophagocytosis is proportional to the sialic acid content (Edwards et al. 1982, Marques et al. 1992). RDP III-3 strains have significantly more capsular sialic acid content than either III-1 or III-2, suggesting that high sialic content may contribute to their increased virulence (Takahashi et al. 1999). However, GBS were able to avoid killing after phagocytosis by monocyte-derived macrophages, even in the presence of opsonic antibody and complement activation (Marodi et al 2000). In addition, using signature-tagged mutagenesis (STM), Jones et al. (2003a) identified a mutation in a gene encoding a penicillin-binding protein

(*ponA*) resulted in decreased virulence in a neonatal rat model of infection and decreased resistance to phagocytosis, suggesting a role for *ponA* in the pathogenesis of GBS. Jones et al. (2003b) identified avirulent mutant with a transposon insertion in *rpoE*, encoding the delta subunit of RNA polymerase using STM. This mutation affected the ability of GBS to inhibit the alternate pathway of complement activation, rendering it more susceptible to phagocytic killing. Poyart et al. (2001) identified a Mn-cofactored superoxide dismutase (SodA) as a virulence factor of GBS. *SodA* mutants were less virulent in a mouse model of infection and more susceptible to bacterial killing by macrophages than wild-type strains. As expected, *sodA* mutants were more susceptible to oxidative stress generated by addition of hydrogen peroxide in culture medium. Survival of oxidative stress following engulfment by professional phagocytes would represent another GBS virulence mechanism involved in surviving the host immune response.

GBS have been shown to rapidly inactivate the complement component C5a by the enzyme C5a-ase (Bohnsack et al. 1991, Hill et al. 1988). Inactivation of C5a reduces PMN recruitment to sites of inflammation and stimulation of PMN phagocytosis, both important factors in immune clearance of GBS (Bohnsack et al. 1997). The work by many investigators suggests that like GBS pathogenesis itself, avoidance of immune clearance is a multi-factoral system. Mutations in seemingly unrelated genes (such as *pon*A, *rpo*E, and *sodA*) resulted in increased susceptibility to phagocytic killing in rat or mouse models of infection (Jones et al. 2003a, 2003b, Poyart et al. 2001), illustrating that surviving the host immune system contributes to GBS pathogenesis. Opsonophagocytosis assays conducted by Brady (1996) suggested that δ/ε-expressing serotype III GBS may be more resistant to intracellular killing by PMNs than δ/ε-negative strains. GBS strain

DL700 (serotype III) was effectively killed following incubation with anti-J48 (serotype III/δε) whole-cell polyclonal rabbit antiserum, while strain J48 (serotype III/δε) itself was not killed in the presence of the same antiserum (Table 1-2). GBS can also survive inside of macrophages and interfere with the bactericidal function of macrophages. Cornacchione et al. (1998) showed that a serotype III strain was able to survive inside murine peritoneal macrophages for up to 48 h and impaired the protein kinase C-dependent transduction pathway. A serotype III strain was also able to survive in a mouse macrophage cell line, and survival was enhanced when entry was opsonin-independent (Valentin-Weigand et al. 1996). Serotype III GBS were also shown to induce apoptosis in murine macrophages (Fettucciari et al. 2000), possibly delaying or hindering the development of a specific immune response.

**Vaccine Development**

Intrapartum prophylaxis has decreased the rate of early-onset, but not late-onset GBS disease in neonates (Schrag et al. 2000). However, the most effective long-term solution for GBS disease, both early- and late-onset, likely lies with the development of an effective vaccine. An ideal vaccine would be protective against all nine serotypes of GBS, but is currently unavailable. Initial work by Lancefield (1934, 1938) showed that protection against GBS infection could be achieved in mice using polyclonal rabbit antiserum made against capsular polysaccharide (CPS), but not group B carbohydrate.

The first experimental vaccine used in a phase I clinical trial was purified serotype III CPS (Baker 1980). Healthy adults safely received the GBS CPS vaccine at a dose range of 10 to 150 μg (Baker et al. 1978, Baker and Kasper 1985, Fischer et al. 1983, Kasper et al. 1983). The safe use of the GBS CPS vaccine in healthy adults led to its

administration to pregnant women at a mean gestation of 31 weeks (Baker et al. 1988). This vaccine was shown to be safe in pregnant women, and there was a direct correlation between maternal and cord blood levels of CPS-specific IgG (Baker et al. 1988). Unfortunately, only 60% of vaccine recipients mounted a specific anti-serotype III GBS CPS response. This result led to attempts to increase the immunogenicity of the CPS.

Coupling of a bacterial CPS to a carrier protein can engage T-cell help and improve immunogenicity, as has been shown with *Haemophilus influenza* type b CPS (Englund et al. 1993). When tested in animals, the first conjugate vaccines of GBS serotype III CPS coupled to tetanus toxoid (TT) elicited opsonically active anti-CPS IgG at levels higher than those achieved by immunization with CPS alone (Lagergard et al. 1990, Wessels et al. 1990). The success of the serotype III CPS-TT conjugate vaccine led to development and preclinical testing of conjugate vaccines with of nine GBS CPSs (Paoletti and Kasper 2002, Paoletti et al. 1992, 1994, Wessels et al. 1990, 1993, 1995). Work in both mice and baboons has also shown that maternal vaccination with GBS glycoconjugate vaccines induces high levels of functionally active IgG that crossed the placenta (Madoff et al. 1994, Paoletti et al. 1996, 2000). Maternal administration of a CPS-TT conjugate vaccine resulted in increased in survival of pups challenged with a lethal dose of serotype-matched GBS compared to pups born to mothers immunized with uncoupled CPS or carrier protein only (Madoff et al. 1994, Paoletti et al. 2000).

This promising work in animal trials led to numerous clinical trials of GBS type III conjugate vaccine, the first with purified type III CPS and monomeric TT (III-TT) (Kasper et al. 1996). The vaccine was administered to nonpregnant women of childbearing age at one of three dosage levels. Controls included age-matched women

immunized with either uncoupled CPS or saline. The vaccination was well tolerated, serum levels of serotype III CPS-specific were dose-dependent, and elevated levels of specific antibody persisted for 26 weeks. In mice and baboons, the administration of alum adjuvant in combination with GBS conjugate vaccines improved the immunogenicity of the vaccine (Guttormsen et al. 1998, Paoletti et al. 1996). Studies have subsequently been conducted in healthy human adults with III-TT adsorbed to aluminum hydroxide gel, but alum did not improve the immune response to the III-TT conjugate vaccine in humans (Paoletti et al. 2001).

Phase 1 and 2 clinical trials have also been conducted with CPS conjugate vaccines prepared with serotypes Ia (Baker et al. 1999), Ib (Baker et al. 1999), II (Baker et al. 2000), and V (Paoletti et al. 1998). To determine if a multivalent vaccine could protect against multiple serotypes, a trial was conducted to evaluate the immune response against two different GBS CPS conjugate vaccines administered in combination (Baker et al. 2003). When GBS serotype II-TT and serotype III-TT were administered together, the immune response to each individual CPS was similar to that achieved using the monovalent vaccines administered singly at the same dose. The bivalent vaccine was well tolerated, and no immune interference was noted from combining the two antigens. More work on the effectiveness of multivalent vaccines needs to be conducted. In the United States, such a strategy would need to include the frequently isolated serotypes (Ia, Ib, II, III, and V), while in Japan, serotypes VI and VIII would need to be included as well (Lachenauer et al. 1999, Paoletti et al. 1999).

Since GBS is a neonatal pathogen, an effective vaccine would need to elicit antibodies that cross the placenta. After showing that administration of a serotype III-TT

conjugate vaccine was safe and effective in healthy women (Kasper et al. 1996), pregnant women were immunized with III-TT (Baker et al. 2001, 2003). The vaccine was safe and well tolerated in these patients, with healthy babies born to each of the vaccinated women. Type III CPS-specific IgG was obtained from maternal serum and cord blood and indicated placental transfer of specific antibody. Importantly, the specific anti-III CPS antisera obtained from 1- and 2-month-old infants were functionally active in opsonophagocytosis assays.

In addition to CPS, several surface proteins have been explored as vaccine candidates and as potential carrier proteins. Immunogenic GBS surface proteins in various phases of development include the α antigen (Gravekamp et al. 1999, Madoff et al. 1994), the β antigen (Gravekamp et al. 1999, Madoff et al. 1994), Rib (Larsson et al. 1996), Sip (Brodeur et al. 2000), and C5a peptidase (Cheng et al. 2001, 2002). Other surface proteins include glutamine synthetase (Suvarov et al. 1997), α-enolase (Pancholi and Fischetti 1998), and Hsp70 (Hammel et al. 1996). Hughes et al. (2002) have also identified major surface proteins, including novel protein vaccine candidates using alkaline phosphatase fusions (Hughes et al. 2003). Both the α and β proteins have been tested for their effectiveness as a carrier protein in a CPS conjugate vaccine with promising results (Gravekamp et al. 1999, Madoff et al. 1994). The α, β, and Rib proteins are each associated with given serotypes. The α and β proteins are more commonly expressed by serotypes Ia, Ib, and II, while Rib and the δ and ε antigens are more commonly associated with serotype III GBS. Therefore, immunization with these antigens would not confer protection against all serotypes. A desirable, but as yet elusive alternative would be to identify an antigen capable of eliciting a more broadly protective

immune response. Although no GBS vaccine is yet available, research suggests that the development of a multivalent GBS vaccine may reduce neonatal GBS disease.

**Specific Aims**

Initial epidemiology and opsonophagocytosis studies have suggested that the GBS δ and ε antigens are markers for a more pathogenic lineage of serotype III, and may contribute to the virulence of this subset of serotype III GBS (Brady et al. 1996). The first specific aim of this study was to isolate and characterize the antigens recognized by δ- and ε-specific antibodies. The second specific aim of this study was to determine genetic components responsible for δ and ε expression. Lastly, the third specific aim of this study was to evaluate the potential role of the δ and ε antigens in the pathogenesis of serotype III GBS. In addition to the work characterizing the δ and ε antigens, a surface-localized glyceraldehyde-3-phosphate dehydrogenase was identified and characterized.

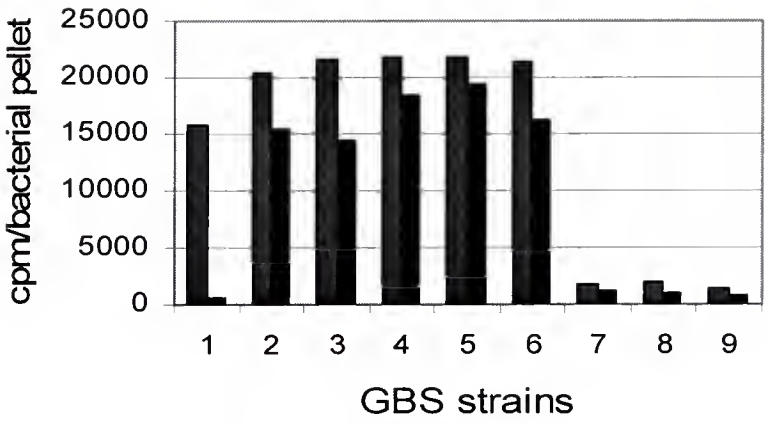



Figure 1-1. Identification of ε reactivity on serotype III, δ-positive group B streptococci by radioimmunoassay. GBS whole cells were incubated with anti-J48 (serotype III/δ) antiserum adsorbed with DL700 (serotype III) (hatched bars) or anti-J48 antiserum adsorbed with DL700 and A909 (solid bars). Results are given as cpm/bacterial pellet. Column 1, A909 (serotype Ic/αβγδ), column 2, J48 (serotype III/δ), column 3, PEH (serotype III/δ), column 4, DL797 (serotype III/δ), column 5, DL769 (serotype III/δ), column 6, DL771 (serotype III/δ), column 7, DL686 (serotype III), column 8, DL775 (serotype III), column 9, DL700 (serotype III).

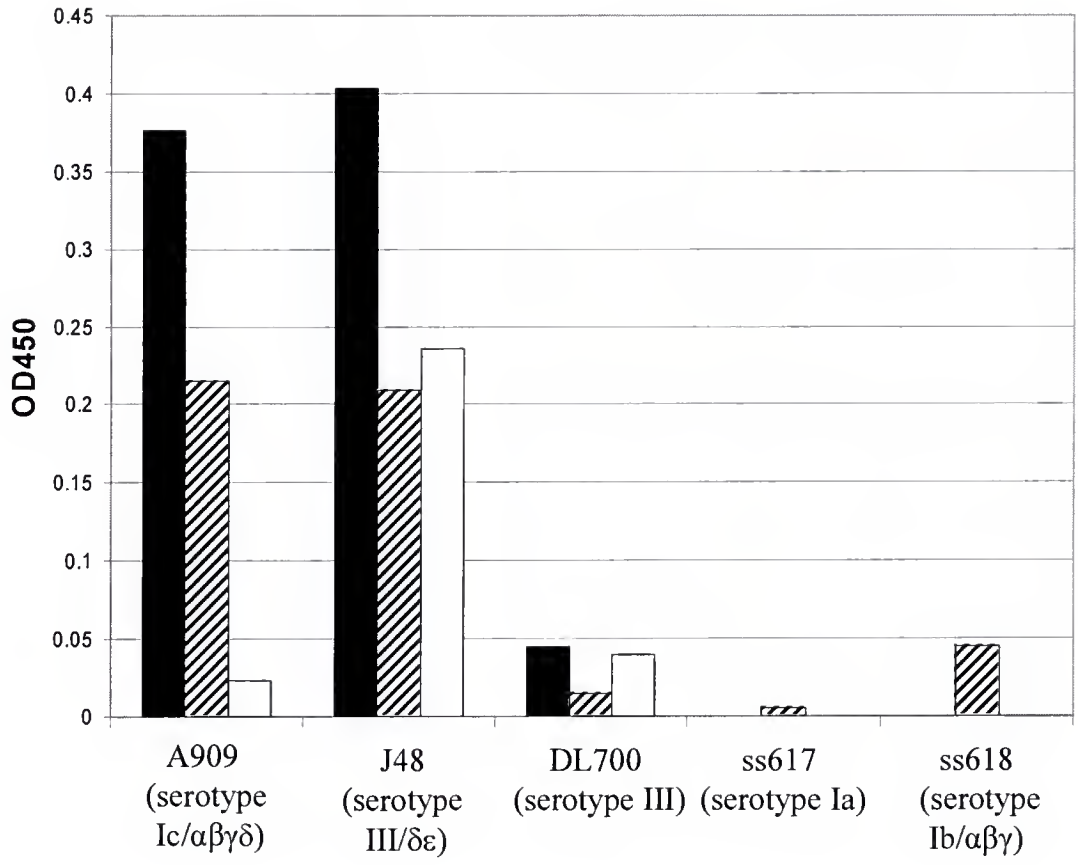



Figure 1-2. Evaluation of anti-δε, anti-δ, and anti-ε reagents with group B streptococcal (GBS) whole cells by ELISA. Solid black bars represent reactivity of anti-δε antiserum. Hatched bars represent reactivity of anti-δ antiserum. Solid white bars represent reactivity of anti-ε antiserum.

Table 1-1. Expression of δ/ε antigens on serotype III group B streptococci isolated from septic vs. colonized sources

| Source of isolates | Number of serotype III strains | Number of serotype III/δε strains | % δ/ε positive serotype III strains |
|---|---|---|---|
| Infant blood<br>Placenta<br>Chorioamniotic fluid | 18 | 15 | 83.3 |
| Colonized infant<br>Colonized mother with healthy infant | 34 | 20 | 58.8 |

Table 1-2. In vitro opsonophagocytic killing of group B streptococci by human peripheral blood leukocytes.

| GBS test strain | Antiserum | CFU-0' | CFU-60' |
|---|---|---|---|
| None | | 0 | 0 |
| J48 | None | 396 | TNTC[1] |
| DL700 | | 352 | TNTC |
| J48 | Unadsorbed | 423 | 356 |
| DL700 | Anti-J48 whole cell | 351 | 40 |
| J48 | Anti-δε specific | 399 | 371 |
| DL700 | | 351 | TNTC |

Results are the mean of triplicate observations. Less than 5% variability was detected between replicate plates. [1]Too numerous to count (>750).

# CHAPTER 2
# ISOLATION AND CHARACTERIZATION OF THE δ AND ε ANTIGENS

## Introduction

Serotypes Ia, Ib, II, III, and V are the most common serotypes isolated from early-onset cases of GBS in the United States (Harrison et al. 1998). Serotype III GBS are responsible for approximately 90% of all late-onset infections (Baker and Edwards 1994), with meningitis being a common clinical manifestation that results in a substantial number of survivors suffering permanent neurological damage. Serotype III GBS are also isolated from cases of adult GBS infection. Therefore, serotype III GBS are a highly pathogenic serotype involved in all manifestations of GBS disease.

In addition to the group- and serotype-specific carbohydrates, a number of studies have been conducted in an attempt to identify and characterize surface components that may contribute to the virulence of serotype III GBS. One of the most well characterized proteins associated with most serotype III GBS is protein Rib (Stalhammar-Carlemalm et al. 1993). Rib is a highly repetitive protein with structural and sequence similarity to the α protein (Wastfelt et al. 1996). Antibodies to protein Rib confer protective immunity in a neonatal mouse model of infection (Stalhammar-Carlemalm et al. 1993). Brady et al. (1988) identified an immunologic reactivity different from Rib and associated with an overlapping but distinct subset of serotype III GBS which was designated δ. Adsorbing a polyclonal rabbit antiserum made against a δ-expressing serotype III strain (J48) with a δ-negative serotype III strain (DL700) rendered the reagent more highly specific for the δ

antigen present on GBS whole cells. When the δ-reactive antiserum was further adsorbed with GBS strain A909 (serotype Ic/αβγδ) to remove antibodies against δ, an additional reactivity remained with all serotype III strains that express the δ antigen. These results indicate that another immunological reactivity is associated with the δ antigen on a serotype III strains. This antigen is absent from strain A909. The newly identified antigen was designated ε.

Preliminary opsonophagocytosis assays and epidemiological data suggest that expression of the δ and ε antigens is a marker for a more pathogenic subset of serotype III GBS and may be involved in the pathogenicity of this subset (Brady et al. 1996). Although δ and ε were discovered based on their antigenic reactivity, the nature of the antigens themselves was unknown. The experiments described in this chapter were designed to isolate and begin to characterize the δ and ε antigens. Initial work was conducted to extract the antigens from GBS whole cells. Anion-exchange chromatography was used to separate δ and ε from other material in sonic extracts and a competition ELISA was used to detect the fluid-phase antigens. To characterize cell-associated δ and ε antigens, whole bacteria were treated with proteinase K or sodium metaperiodate. Column chromatography fractions enriched in δ and ε were also analyzed by Western immunoblot using appropriate antisera to identify reactive material. In an attempt to generate a more ε-specific polyclonal antiserum, ε-containing material present in a sonic extract and eluted from an SDS-polyacrylamide gel was used as an immunogen. As a basis for comparison, corresponding material from an ε-negative serotype III strain was also used as an immunogen.

## Materials and Methods

### Bacterial Strains and Growth Conditions.

GBS strains used in this study were A909 (serotype Ic/αβγδ), J48 (serotype III/δε), J52 (serotype III/δε), PEH (serotype III/δε), COH1 (serotype III/δε), DL1104 (serotype III), DL700 (serotype III), and ATCC 12403 (serotype III). Strain A909 (serotype Ic/αβγδ) was obtained from Richard Facklam at the Centers for Disease Control and Prevention (Atlanta, GA, USA). Strains J48 (serotype III/δε), J52 (serotype III/δε), and PEH (serotype III/δε) were clinical isolates from Shands Hospital, University of Florida. Strain COH1 (serotype III/δε) was a generous gift from Dr. Craig Rubens (Children's Hospital Regional Medical Center, Seattle, WA, USA). Strains DL1104 (serotype III) and DL700 (serotype III) were clinical isolates kindly provided by Dr. Daniel Lim (University of South Florida, Tampa, FL, USA. Strain ATCC12403 (serotype III) was obtained from American Type Culture Collection (Manassas, VA). All streptococci were grown to late exponential phase in Todd-Hewitt Broth (Becton Dickinson, Cockeysville MD) at 37°C without shaking.

### Generation of Polyclonal Anti-δε, Anti-δ, and Anti-ε Antiserum

Polyclonal anti-δε, anti-δ, and anti-ε rabbit antisera were made by immunizing New Zealand white rabbits with formalin-killed whole cells, followed by adsorption with appropriate GBS strains. To make an anti-δε reagent, a polyclonal rabbit antiserum was generated against J48 (serotype III/δε) whole cells. Cells from a 200 mL stationary phase culture were fixed by treatment with 10 mL of 4% formalin, 0.85% saline solution at 4°C overnight. The density of the immunogen was adjusted to an $OD_{660}$ of 0.4. A rabbit was injected intravenously with 0.5mL of the bacterial suspension 3 times/week for 1 week

and then with 1 mL 3 times/week for 3 weeks. The rabbit was rested for 3 weeks and then injected with 1 mL 3 times/week for 2 weeks. The rabbit was exsanguinated by cardiac puncture under anesthesia. Serum was stored at -20°C. This polyclonal rabbit anti-J48 antiserum was made δε-specific by adsorption with GBS strain DL700 (serotype III). PBS-washed DL700 whole cells (from a 100 mL overnight culture) were rotated with an aliquot (1 mL) of the antiserum end-over-end at 4°C for 1 h to remove antibodies to the serotype III and group B polysaccharides, as well as any other common surface antigens such as protein Rib (Stalhammar-Carlemalm et al. 1993). This procedure was repeated using fresh cells until reactivity against the adsorbing strain was eliminated. Appropriate adsorption and specificity was indicated by reactivity with GBS strains J48 and A909, but not DL700 as determined by whole cell ELISA. To make an ε-specific polyclonal antiserum, the anti-δε antiserum was further adsorbed with GBS strain A909 (serotype Ic/αβγδ), a serotype Ia strain that expresses δ, but not ε. The adsorbed polyclonal rabbit antiserum was determined to be ε-specific if it was reactive with strain J48, but not strains DL700 or A909 by whole cell ELISA. To make polyclonal anti-δ antiserum, rabbits were immunized with GBS strain A909. Polyclonal rabbit anti-A909 antiserum was made δ-specific by adsorbing the antiserum with strains ss617 (serotype Ia) and ss618 (serotype Ib/αβγ) to remove antibodies to all known antigens except δ. The resulting reagent was determined to be δ-specific if it was reactive with strains A909 and J48, but not strains ss617, ss618, or DL700. A representative ELISA assay showing specificity of each of the adsorbed antiserum is shown in Figure 1-2.

**Extraction of δ and ε Antigens**

Extracts of GBS strains J48 (serotype III/δε) and DL700 (III) were made by sonicating bacteria three times for 30 s in 20 mM Tris-HCl pH 8.0 with P-800 Potter's glass beads (1:1:1, wet weight per volume per weight) on ice with a Sonic 300 Dismembrator (ARTEK Systems Corporation, Farmingdale, NY). The glass beads and bacterial debris were removed from the sonicated mixture by centrifugation at 10 × *g* for 5 min. Wall membranes were removed in a Beckman L7-55 Ultracentrifuge at >100,000 × *g* for 2 h. The non-pelleted material was collected and filtered through 0.2 mm Acrodiscs (Gelman Sciences, Ann Arbor, MI).

**Detection of δ and ε Antigens by Competition ELISA**

GBS strain J48 (serotype III/δε) whole cells were used to coat Costar High Binding plates (Costar, Corning, NY). Sample wells were coated overnight at 4°C in a moist chamber with 100 µl of 0.1M carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide and approximately $10^7$ streptococcal whole cells. The plates were blocked with PBS containing 0.03% Tween-20 (PBS-Tw). Test wells were incubated with 100 µl of bacterial sonic extract (serial 3-fold dilutions) and 100 µl of anti-δε antiserum (1:100 dilution in PBS) for 2 h at 37°C. After washing, wells were incubated with horseradish peroxidase (HRP)-labeled goat-anti-rabbit IgG at a 1:1000 dilution (ICN Biomedicals, Inc, Aurora, OH) for 2 h at 37°C. Plates were washed, and developed with o-phenylenediamine in 0.1M citric acid/0.2M sodium phosphate buffer in the presence of 0.03% hydrogen peroxide. $OD_{450}$ was determined using a Model 550 microplate reader (Bio-Rad, Hercules, CA). Inhibition was determined by comparing $OD_{450}$ values of test wells with $OD_{450}$ values of control wells containing 100 µl of buffer only instead of a

column fraction. Results are given as % inhibition. Percent Inhibition = 1 – [ $OD_{450}$ (average of wells containing inhibitor and antibody) – (secondary antibody only background)] / [$OD_{450}$ (average of wells containing antibody and buffer only) – (secondary antibody only background) ] × 100.

**Anion-Exchange Chromatography**

Bacterial sonic extracts were diluted 1:3 in 20 mM Tris-HCl, pH 8.0, and loaded onto an UNO-Q1 (Bio-Rad, Hercules, CA) anion-exchange column. Unbound material was removed from the column by washing with 2.5 column volumes of 20 mM Tris-HCl pH 8.0. Bound molecules were eluted from the column using a stepwise gradient (5 mL for each step) of 0-1 M NaCl of 0.05 M NaCl increments in 20 mM Tris-HCl pH 8.0.

**Detection of δ and ε in Individual Anion-Exchange Column Fractions**

To detect δ, GBS strain A909 whole cells were used to coat Costar High Binding plates (Costar). For ε detection, GBS strain J48 was used. The presence of the δ and/or ε antigens was determined by competition ELISA as described above.

**Proteinase K and Sodium Metaperiodate Sensitivity of Cell-Associated δ Antigen**

GBS strain A909 (serotype Ic/αβγδ) whole cells were used to coat Costar High Binding plates (Costar) as described above. Test wells were incubated with 100 µl of either PBS or PBS containing 0.05% proteinase K (Sigma Chemical Co.) for 2 h at room temperature, or with 100 µl of either 0.05 M sodium acetate buffer, pH 5.5 or acetate buffer containing 0.05 M sodium metaperiodate (Sigma Chemical Co.) for 16 h at room temperature. After treatment, the proteinase K or sodium metaperiodate was removed from the wells by multiple washes with PBS-Tw. The plates were blocked with PBS-Tw, and incubated with 100 µl of anti-δε polyclonal antiserum (1:100 dilution in PBS) for 2 h

at 37°C, washed and developed as described above. The presence of anti-ε antibodies in the anti-δε reagent was irrelevant in these experiments since A909 does not express this antigen.

**Enrichment of δ-Specific Antibodies by Immunoprecipitation**

An immunoprecipitation method was used in an attempt to increase the concentration of anti-δ antibodies in the polyclonal anti-δε rabbit sera. Whole cells from replicate 10 mL overnight cultures of A909 (serotype Ic/αβγδ) were harvested by centrifugation. Ten milliliters of 4% formalinized 0.85% saline was added to the cells, mixed, and stored overnight at 4°C. Cells were checked for lack of viability after 24 h by streaking on Todd Hewitt Broth plates. Five-hundred microliters of anti-δε antiserum was incubated with one aliquot of GBS strain A909 whole cells and rotated for 2 h at 4°C. Cells were pelleted, and the supernatant was transferred to another aliquot of A909 whole cells. This procedure was repeated 10 times. Pelleted cells were incubated with 200 µl of 0.5 M acetic acid, 0.15 M NaCl to elute bound anti-δ antibodies. Eluted antibodies were dialyzed against PBS and concentrated with a Sieze® X Protein G Immunoprecipitation Kit (Pierce Biotechnology, Rockford IL) according to the manufacturer's instructions.

**Detection of the δ Antigen by Western Immunoblot**

Proteins contained in anion-exchange column fractions #14 from J48 and DL700 (Figure 2-2) were separated on duplicate 7.5% SDS-polyacrylamide gels, electroblotted onto PVDF (NEN™ Life Science Products, Boston, MA) or nitrocellulose membranes (Protran®, Scleicher & Schuell, Keene, NH) and blocked with PBS-Tw. The PVDF membrane was incubated with AuroDye Forte™ (Amersham, Piscataway, NJ). The nitrocellulose was incubated with antiserum enriched for anti-δ antibodies (1:500)

overnight at room temperature. After washing, the blot was incubated with HRP-labeled goat-anti-rabbit IgG at a 1:1000 dilution (ICN Biomedicals, Inc) for 2 h at room temperature, washed, and developed with ECL™ detection reagents (Amersham Life Sciences, San Francisco, CA). An immunoreactive band (~150 kDa) present in the J48 sample was excised from the PVDF membrane and subjected to N-terminal sequencing by the Interdisciplinary Center for Biotechnology Research (ICBR), University of Florida, Gainesville, USA using automated Edman chemistry.

In addition to analysis of chromatography fractions, cells from overnight 10 mL cultures of a panel of δ surface-positive and δ surface-negative GBS strains were centrifuged, washed with l mL PBS, and resuspended in 200 µl 1X SDS-sample buffer. Resuspended cell suspensions were boiled for 10 min, and debris removed by centrifugation. Proteins present in the supernatants were analyzed by Western immunoblot using antiserum enriched for anti-δ antibodies as described above. Immunoreactive bands present in samples from δ-positive (~25 kDa) and δ-negative strains (~35 kDa) were excised from the PVDF membrane and subjected to N-terminal sequencing by the ICBR.

**Detection of the ε Antigen by Western Immunoblot**

Material contained in an ε-containing anion-exchange column fraction #14 from J48 (Figure 2-2) was analyzed by Western immunoblot as described above for detection of the δ antigen with the adsorbed anti-ε antiserum (1:1000) as the primary detection reagent. The corresponding column fraction from DL700 served as a negative control.

**Proteinase K and Sodium Metaperiodate Sensitivity of Cell-Associated ε Antigen**

Experiments were performed as described above for evaluation of the δ antigen except that GBS strain J48 (serotype III/δε) was used instead of A909 and anti-ε antiserum (1:100) was used as the detection reagent.

**Sodium Metaperiodate Sensitivity of Isolated ε Antigen by Western Immunoblot**

Material contained in an ε-containing anion-exchange column fraction was separated on 7.5% SDS-polyacrylamide gels and electroblotted onto duplicate nitrocellulose membranes (Protran®, Scleicher & Schuell). Before being blocked with PBS-Tw, the membranes were treated with 0.05 M sodium acetate buffer pH 5.5 or acetate buffer containing 0.05 M sodium metaperiodate (Sigma Chemical Co.) for 16 h at room temperature. After blocking, the treated and untreated membranes were incubated with anti-ε polyclonal antiserum (1:1000) overnight at room temperature. After washing, the blots were incubated with HRP-labeled goat-anti-rabbit IgG at a 1:1000 dilution (ICN Biomedicals, Inc) for 2 h at room temperature, washed, and developed with ECL™ detection reagents (Amersham Life Sciences).

**Gel Purification of ε-Containing Material from an SDS-Polyacrylamide Gel: Comparison of Comparable Material from an ε-Negative Strain**

Material from bacterial sonic extracts of GBS strains J48 (serotype III/δε) and DL700 (serotype III) was separated on replicate 7.5% preparatory SDS-polyacrylamide gels. A 2 mm gel-slice (above the 250-kDa protein standard) was excised from each gel. The ε antigen was shown previously by Western immunoblot to migrate at this apparent molecular mass. Each gel slice was finely minced with a razor blade and placed in a 1.5 mL tube. Material was eluted from the minced gel slices with 500 µl of 50 mM

ammonium bicarbonate pH 7.4, 1% SDS and rocking for 2 h at 37°C. The sonic extracts and gel-eluted materials were analyzed by Western immunoblot as described above with anti-ε antiserum, anti-δ antiserum, and anti-serotype III antiserum (Accurate Chem., Westbury, NY) as primary detection reagents. The gel-eluted materials from each strain were assayed for the presence of group B carbohydrate antigen by latex agglutination (Phadabact®, Boule Diagnostics, Huddinge, Sweden). The gel-eluted materials were also analyzed for total amino acid content by the ICBR, and for carbohydrate content by the Complex Carbohydrate Research Center, University of Georgia, Athens USA, supported in part by the Department of Energy-funded (DE-FG09-93ER-20097) Center for Plant and Microbial Complex Carbohydrates.

Gel-purified materials from J48 and DL700 were used as immunogens to produce polyclonal rabbit antisera (Lampire Biological Laboratories, Pipersville, PA). Rabbits were injected with 100 µl of each eluted sample mixed with 100 µl of Incomplete Freund's Adjuvant on days 1, 7, 14, and 28. On day 42, a 2 mL test bleed was removed. Rabbits were injected again on days 56 and 84, and a termination bleed was obtained on day 98.

Sonic extracts of representative of δε-positive and δε-negative GBS strains were separated on duplicate 7.5% SDS-polyacrylamide gels, electroblotted onto duplicate nitrocellulose membranes (Protran®, Scleicher & Schuell), and blocked with PBS-Tw. Each membrane was incubated with either polyclonal rabbit antiserum made against ε-containing gel-eluted material from J48 (1:1000) or comparable material from DL700 sonic extracts. After washing, blots were incubated with HRP-labeled goat-anti-rabbit

IgG at a 1:5000 dilution (ICN Biomedicals, Inc) for 2 h at room temperature, washed, and developed with ECL™ detection reagents (Amersham Life Sciences).

## Results

### Extraction and Detection of the δ and ε Antigens

Sonic extracts of strain J48 (serotype III/δε) and DL700 were prepared and the presence of the δ and ε antigens evaluated by competition ELISA. This approach was chosen because the adsorbed antisera recognize the antigens under study specifically on whole bacterial cells, but as polyclonal reagents they also recognize additional antigens present in cellular extracts.

The J48 (serotype III/δε) sonic extract inhibited binding of anti-δε antiserum to J48 (serotype III/δε) whole cells in a dose-dependent manner whereas an extract of DL700 (serotype III) did not (Figure 2-1). Similar results were observed when anti-δ or anti-ε antisera were used, confirming the presence of both antigens in the extract of J48 but not DL700 (data not shown).

### Enrichment for the δ and ε Antigens by Anion-Exchange Chromatography

Bacterial sonic extracts were diluted in 20 mM Tris-HCl, pH 8.0, and loaded onto an UNO-Q1 (Bio-Rad, Hercules, CA) anion-exchange column. Comparisons of representative anion-exchange column runs from J48 (serotype III/δε, Figure 2-2, Panel A) and DL700 (serotype III, Figure 2-2, Panel B) are shown. While the δ and ε antigens are detectable in the J48 but not the DL700 starting material, the elution profiles of these two strains are remarkably similar.

The competition ELISA used to detect the presence of δ and ε in the J48 sonic extract was also used to detect the antigens in individual anion-exchange column

fractions (Figure 2-3). Material eluted from the column with 0.2M NaCl (fractions 13-17) from J48 inhibited anti-δε antiserum reactivity with A909 (serotype Ic/αβγδ) whole cells (Panel A), while other column fractions from J48 and column fractions from DL700 did not. Anti-ε reactivity with J48 whole cells was also inhibited by fractions 13-17 from J48 (Panel B), indicating that both δ and ε co-eluted from the anion-exchange column in a peak with 0.2 M NaCl. These fractions were subsequently demonstrated to also contain the group B and serotype III polysaccharides (data not shown).

**Proteinase K Sensitivity of the δ Antigen on A909 Whole Cells.**

To determine if the δ antigen was sensitive to proteolysis, A909 (serotype Ic/αβγδ) whole cells were treated with proteinase K or buffer only (Figure 2-4). Treatment of A909 whole cells with proteinase K substantially reduced the binding of anti-δε antiserum compared to treatment with buffer only. This suggests that the δ antigen is comprised of protein.

**Sodium Metaperiodate Sensitivity of the δ Antigen on A909 Whole Cells.**

To further characterize the δ antigen, A909 whole cells were also treated with sodium metaperiodate or buffer only (Figure 2-5). Treatment of A909 whole cells with sodium metaperiodate had no discernible effect on anti-δε binding suggesting that δ does not have a carbohydrate component.

**Evaluation of a δ-Containing Anion-Exchange Fraction by Western Immunoblot**

Material from an anion-exchange column fraction from strain J48 (serotype III/δε) that contained the δ antigen as determined by competition ELISA was separated by SDS-PAGE and transferred to either nitrocellulose or PVDF. The nitrocellulose membrane was incubated with antiserum enriched for anti-δ antibodies by immunoprecipitation with

strain A909 (serotype Ic/αβγδ) (Figure 2-6). An immunoreactive band migrating at ~150 kDa was present in the column fraction from J48, but not the corresponding column fraction from DL700. The ~150-kDa J48-specific band present on a replicate PVDF membrane stained for protein was subjected to N-terminal sequencing by the ICBR using automated Edman chemistry. The sequence obtained was AVFEKVQEIIVEXLGKD. BLAST analysis (Altschul et al. 1997) demonstrated homology (16/17 amino acids) with the deduced amino acid sequence (amino acids 2-18 of 74 total) of *gbs0332* (Glaser et al. 2002), an acyl-carrier protein of GBS.

**Western Immunoblot of SDS-Extracted Material with Anti-δ-Enriched Antiserum**

SDS-extracted material from a panel of GBS strains was tested to identify δ-reactive bands present in material extracted by a method other than sonication. SDS-extracted material from representative δ-positive and δ-negative GBS strains was separated by SDS-PAGE and transferred to either nitrocellulose or PVDF. The nitrocellulose membrane was incubated with the antiserum enriched for anti-δ antibodies by immunoprecipitation with strain A909 (serotype Ic/αβγδ) (Figure 2-7). An immunoreactive band migrating at ~25 kDa (left arrow) was observed for strain A909 and the four serotype III/δε-positive strains tested. In contrast, an immunoreactive band migrating at ~35 kDa was observed for serotype III/δε-negative strains (right arrow). Both the ~25-kDa and 35 kDa bands were excised from a replicate PVDF membrane stained for protein, and subjected to N-terminal sequencing by the ICBR using automated Edman chemistry. The amino acid sequence obtained from the 25-kDa band from δ-positive strains was ADVF(E/D)KVQ(E/G)IXDEXL(G/Q). The amino acid sequence obtained from the 35 kDa band from δ-negative strains was

A(I/V)FE(D/K)VQ(A/E)(R/I)(E/X)V(L/E)RK. BLAST analysis (Altschul et al. 1997) of both anti-δ immunoreactive bands demonstrated homology (amino acids 2-17 and 2-15, respectively) with the deduced amino acid sequence of *gbs0332* (Glaser et al. 2002), the same acyl-carrier protein associated with the 150-kDa anti-δ reactive band observed upon analysis of anion-exchange column fraction #14 of J48 sonic extract.

**Visualization of ε by Western Immunoblot**

Material from a column fraction that contained ε as determined by competition ELISA was electrophoresed on a 7.5% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with anti-ε antiserum to identify immunoreactive bands present in the J48 (serotype III/δε) sample, but not the corresponding column fraction from DL700 (serotype III) (Figure 2-8). A prominent band migrating at >250 kDa reactive with anti-ε antiserum and present only in the column fraction from J48 was putatively identified as ε. This material was subsequently eluted from an SDS-gel slice and was able to inhibit anti-ε binding to J48 whole cells by competition ELISA, confirming that the high molecular weight material contained the ε antigen (data not shown).

**Proteinase K Sensitivity of the ε Antigen on J48 Whole Cells.**

To determine whether the ε antigen was sensitive to proteolysis, J48 (serotype III/δε) whole cells were treated with proteinase K or buffer only (Figure 2-9). The ability of anti-ε antiserum to bind to proteinase K-treated but not buffer-treated J48 (serotype III/δε) whole cells was substantially reduced, suggesting a protein component of the ε antigen.

**Sodium Metaperiodate Sensitivity of the ε antigen from J48**

To determine if carbohydrate moieties contribute to the ε antigen, J48 (serotype III/δε) whole cells were treated with sodium metaperiodate or buffer only (Figure 2-10). Treatment of J48 whole cells with sodium metaperiodate, but not buffer only, reduced anti-ε binding by over 50%, suggesting that in addition to protein, the ε antigen is comprised of a carbohydrate component as well.

Western immunoblot was also used to determine whether reactivity of ε-containing material was destroyed by treatment with sodium metaperiodate. Increasing amounts of material contained in an ε-containing anion-exchange column fraction from J48 were separated on 7.5% SDS-polyacrylamide gels and electroblotted onto duplicate nitrocellulose membranes. Membranes were incubated with sodium metaperiodate or buffer only before incubation with anti-ε antiserum (Figure 2-11). Treatment of the ε antigen immobilized on a nitrocellulose membrane with sodium metaperiodate eliminated the ability of anti-ε antibodies to bind to the >250-kDa immunoreactive band.

**Gel-Purification ε-Containing Material from an SDS-Polyacrylamide Gel: Comparison of Comparable Material from an ε-Negative Strain**

Western immunoblot was used to localize anti-ε reactive material from J48 (serotype III/δε) to >250 kDa on 7.5% SDS-polyacrylamide gels, although no protein stained bands were detected in this region of a replicate gel. Assuming the antigen would be present, ε-containing material was extracted from an SDS-polyacrylamide gel of a sonic extract of J48 using the 250-kDa protein standard as a guide. As a basis for comparison, similar sized material was gel-purified from a sonic extract of DL700 (serotype III) as well. The extracted materials were analyzed by Western immunoblot

(Figure 2-12), incubating replicate membranes with anti-δ, anti-ε, and anti-serotype III antisera. Anti-ε antiserum bound only to the gel-purified material from J48. Anti-δ and anti-serotype III antiserum did not bind to the gel-purified material from either strain. Gel-purified material from both strains demonstrated reactivity with anti-group B carbohydrate antibodies as determined by latex agglutination (Phadabact®, Boule Diagnostics, Huddinge, Sweden). The samples were not reactive with antibodies against groups A, C, D, or G carbohydrates by latex agglutination (data not shown). The gel-purified materials were subjected to total amino acid and carbohydrate composition analysis and both contained the polysaccharides rhamnose, mannose, galactose, and glucose. Rhamnose and galactose are known components of the group B streptococcal carbohydrate antigen, while mannose and glucose are not. Amino acid analysis suggested the presence of either a glycine- or serine-rich protein in each sample (data not shown).

Gel-purified ε-containing material from J48 and corresponding material from DL700 containing group B carbohydrate were used as immunogens to produce polyclonal rabbit antisera. These antisera were used in Western immunoblot analysis of SDS-boiling cell extracts from representative strains of GBS (Figure 2-13). The polyclonal rabbit antiserum made against gel-purified material from J48 bound to the >250-kDa band known to represent the ε antigen (arrow). The anti-DL700 rabbit antisera made against corresponding material was not reactive with this >250-kDa band and did not recognize any unique bands in SDS-extracted material from serotype III/δε-negative GBS. To remove antibodies against common determinants, the polyclonal antiserum generated against gel-purified material from J48 was adsorbed with DL700 whole cells. The adsorbed reagent retained reactivity with J48 whole cells, but binding to DL700

whole cells was drastically reduced indicating that the adsorbed antiserum contained antibodies against the ε antigen (data not shown). To determine if the polyclonal antiserum made against the gel-purified material from DL700 recognized any antigens unique to serotype III/δε-negative strains and not expressed by serotype III/δε-positive strains, this antiserum was adsorbed with J48 whole cells. The reactivity of the adsorbed reagent was eliminated against both DL700 and J48 indicating the gel-purified material from DL700 did not contain any additional antigens not also contained in the corresponding sample from J48 (data not shown).

## Discussion

To isolate and characterize the δ and ε antigens, multiple techniques were employed to determine an efficient method of extraction, including treatment with mutanolysin, boiling whole cells in SDS, sonication, and autoclaving whole cells (Rantz and Randall 1955). Western immunoblot analysis using anti-δ and anti-ε specific reagents and SDS-polyacrylamide gels stained with Coomassie Brilliant Blue protein stain suggested that sonication provided extraction of the desired antigens and the highest total protein yield (data not shown). Therefore, sonication was chosen as a method for generating starting material for subsequent isolation and purification techniques. A major drawback to this method was that a number of irrelevant cytosolic proteins were extracted as well. In spite of this drawback, sonication was used because relatively large amounts of extract could be generated without substantially altering structures that may have been modified by mutanolysin treatment or by autoclaving whole cells.

Although adsorbed polyclonal rabbit antisera detect δ and ε specifically on the GBS cell surface, these reagents recognize additional antigens contained in cellular extracts.

Therefore, a competition ELISA was used to detect fluid-phase δ and ε antigens (Figure 2-1). The binding of anti-δε antiserum to J48 (serotype III/δε) whole cells was inhibited in a dose-dependent manner by a sonic extract of this strain but not by a sonic extract of DL700 (serotype III). Similar results were observed with anti-δ and anti-ε antisera (data not shown), indicating that sonication of GBS whole cells was effective in extracting both the δ and ε antigens.

Because a sonic extract of J48 (serotype III/δε) whole cells contained δ and ε this material, in comparison with a sonic extract of DL700 (serotype III), was used as the starting material for anion-exchange chromatography in an attempt to isolate and identify these antigens (Figure 2-2). Elution of bound material with a linear gradient of increasing NaCl concentrations resulted in 2 broad peaks. One small peak was observed spanning NaCl concentrations of 0-0.25 M, while one large peak encompassed 0.25-2 M NaCl (data not shown). Multiple smaller fractions corresponding to more well-defined peaks were eluted with a stepwise gradient of 0-2 M NaCl. Therefore, this approach was used for enrichment of the δ and ε antigens.

A competition ELISA was used to detect δ and ε contained in individual anion-exchange column fractions (Figure 2-3). To detect δ, column fractions were tested for inhibition of anti-δε antiserum binding to A909 (serotype Ic/αβγδ) whole cells. This approach was chosen because the adsorbed anti-δ antiserum was of low titer and reserved for other experiments. Although the anti-δε antiserum contains antibodies that recognize ε, A909 (serotype Ic/αβγδ) does not express ε, so these antibodies are irrelevant to the assay. Inhibition of anti-ε antiserum binding to J48 (serotype III/δε) was used to identify fractions containing ε. Both δ and ε eluted with 0.2 M NaCl (column fractions #13-17) in

anion-exchange column runs of J48 sonic extracts. In addition, material reactive with antibodies against the serotype III and group B polysaccharides were detected in the same fractions (data not shown), suggesting that multiple surface structures co-purify by this technique. The presence of additional cellular components in these column fractions has not been evaluated.

To determine if the δ antigen was sensitive to protease treatment, A909 (serotype Ic/αβγδ) whole cells were treated with proteinase K. Proteinase K has broad substrate specificity and cleaves at the carboxyl side of hydrophobic, aliphatic, and aromatic amino acids. Therefore, proteinase K should digest most surface proteins of GBS. Treatment of A909 whole cells with proteinase K substantially reduced binding of the anti-δε antiserum (Figure 2-4), suggesting that the δ antigen was proteinaceous.

Because group B and serotype III polysaccharide antigens were detected in column fractions also containing δ, sensitivity of δ to treatment with sodium metaperiodate was evaluated (Figure 2-5). Sodium metaperiodate cleaves adjacent hydroxyl groups in carbohydrate molecules. Anti-δε antiserum was able to bind to A909 (serotype III/δε) whole cells after treatment with sodium metaperiodate, which suggests that carbohydrate does not contribute to the δ antigen. The possibility still exists that a carbohydrate moiety unaffected by sodium metaperiodate is a component of the δ antigen.

Because adsorbed anti-δ antiserum derived from c-protein antiserum made against strain A909 (Ic/αβγδ) was very low titer, an attempt was made to increase the concentration of δ-specific antibodies contained in anti-δε antiserum made against J48 (serotype III/δε). To this end, the anti-δε antiserum was reacted with A909 (Ic/αβγδ) whole cells, unbound antibodies washed away, and bound antibodies eluted from the

whole cells. This reagent was used to identify immunoreactive bands present in a δ-containing anion-exchange column fraction from J48 (serotype III/δε), by comparison with the comparable column fraction from DL700 (serotype III) (Figure 2-6). A 150-kDa immunoreactive band was present in the column fraction from J48, but not from DL700 and was determined to have N-terminal sequence homology to a GBS acyl-carrier protein (ACP). ACP is a small (~10-kDa), common enzyme known to carry substrates to six separate enzymes for sequential processing during fatty acid biosynthesis (Wakil et al. 1983). In addition to its role in fatty acid biosynthesis, it has also been shown to be involved in membrane-based oligosaccharide synthesis (Therisod et al. 1986, Tang et al. 1997) and lipid biosynthesis (Rock and Jackowski 1982) in *E. coli*. ACP is also involved in the formation of capsular polysaccharide of rhizobia (Epple et al. 1998). It is unknown why the GBS ACP was isolated from a band migrating at ~150 kDa when ACP itself is only predicted to be 10 kDa. The most likely explanation is that ACP is bound to another molecule. This would be consistent with the recent findings of Gully et al. (2003), who showed that ACP from *E. coli* could interact with proteins in addition to those necessary for fatty acid biosynthesis. Furthermore, ACP formed a disulfide bond with IscS, a protein involved in the synthesis of sulfur-containing molecules. Additional experiments will need to be completed to determine if ACP is coupled to GBS proteins other than the putative δ antigen contained in the ~150-kDa band observed by Western immunoblot.

In a further attempt to identify the GBS δ antigen, SDS-extracted material from representative δ-positive and δ-negative GBS strains were also analyzed by Western immunoblot and incubated with immunoprecipitated, δ-enriched antiserum (Figure 2-7). A ~25-kDa immunoreactive band present in samples from all δ-surface positive GBS also

demonstrated N-terminal sequence homology to ACP. An immunoreactive band migrating at ~35 kDa present in samples from all δ-surface negative strains also demonstrated N-terminal sequence homology to ACP. Demonstration of ACP migrating at two different molecular weights depending on whether the GBS strain expressed δ on its surface was an interesting observation. However, these results are difficult to interpret without further experimentation. Recombinant ACP was not reactive with anti-δ antibodies indicating that ACP itself does not represent δ. Because the anti-δ reagent likely recognizes non-δ moieties present in cellular extracts, it is not possible to determine whether reactivity of the anti-δ reagent with the ~35-kDa band in δ-negative strains represents a non-δ ACP-associated molecule or whether this band represents a δ variant not exposed on the surface of this subset of strains.

Epsilon was identified by Western immunoblot of an anion-exchange column fraction of J48 (serotype III/δε) as a high molecular weight antigen (>250-kDa) that was not detectable by conventional protein stains (Figure 2-8). Binding of anti-ε antiserum to J48 was decreased by treatment with proteinase K (Figure 2-9) and sodium metaperiodate (Figure 2-10), suggesting that both protein and carbohydrate components contribute to the ε antigen. Treatment of J48 whole cells with proteinase K completely eliminated binding of anti-ε antibodies. Although treatment with proteinase K would destroy the protein backbone of a glycoprotein antigen, such treatment could also extract the antigen. Treatment of J48 whole cells with sodium metaperiodate reduced antibody binding by approximately 50% suggesting that ε most likely represents a glycoprotein antigen.

Anti-ε antiserum no longer bound to ε-containing material after sodium metaperiodate treatment of blotted antigens on nitrocellulose (Figure 2-11). This

complete ablation on anti-ε reactivity contrasted with the results observed with whole cells where antibody binding decreased by only 50% after treatment with sodium metaperiodate. It is possible that the protein component recognized by anti-ε on whole cells was denatured and no longer recognized by anti-ε antibodies on the Western immunoblot.

The >250-kDa ε-containing material from the J48 (serotype III/δε) sonic extract and the corresponding material from DL700 (serotype III) were eluted from SDS-polyacrylamide gels and analyzed by Western immunoblot (Figure 2-12). Eluted material from J48, but not DL700, was recognized by anti-ε, but not anti-δ or anti-serotype III antisera. The material eluted from J48, but not DL700 was able to inhibit anti-ε antiserum binding to J48 whole cells in a competition ELISA supporting the hypothesis that this high-molecular weight antigen is ε (data not shown). Eluted materials from both strains were reactive with a group B carbohydrate latex agglutination typing reagent. Since ε and anti-group B reactive material co-purify by anion-exchange chromatography and are in the same >250-kDa eluted material, one possibility is that these two entities are closely associated on the cell surface and sonication or SDS-extraction do not separate the two. Another possibility is that both entities are of similar molecular weight and co-migrate by SDS-PAGE. Carbohydrate analysis of the eluted material from both strains demonstrated the presence of rhamnose, mannose, galactose, and glucose in similar ratios for each strain (4:1:1:6, data not shown). The group B carbohydrate consists of rhamnose, *N*-acetylglucosamine, galactose, and glucitol (Michon et al. 1988). Therefore, in addition to having rhamnose and galactose in common with the group B carbohydrate, the eluted material from each strain also contained mannose and

glucose. The absence of N-acetylglucosamine and glucitol from the gel-purified materials of strains J48 and DL700 did not preclude reactivity with the group B specific typing reagent. This suggests that immunologic detection of group specific determinants do not depend on the presence of the complete native antigen.

Preliminary amino acid analysis of the ε-containing gel-purified material from J48 and corresponding material from DL700 suggested the presence of a glycine-rich or possibly serine-rich protein. Examination of the genome database of a serotype III strain (Glaser et al. 2002) revealed no glycine-rich proteins. The genome did however, contain an open reading frame (ORF) with a deduced amino acid sequence of a serine-rich protein. Since serine residues are potential sites for either *N*– or *O*-linked carbohydrates, a serine-rich protein could serve as a backbone for glycosylation and account for the presence of sugars found in each sample.

In an attempt to generate a more specific and higher titer anti-ε antiserum, eluted ε-containing material from J48 (serotype III/δε) was used to immunize rabbits. For comparison, an antiserum was generated against the corresponding material from DL700 (serotype III) as well. Both polyclonal antisera were incubated with SDS-extracted antigens from numerous GBS strains (Figure 2-13). The antiserum made against the J48 material bound to the >250-kDa antigen present in all ε-positive, but not ε-negative, strains. The antiserum made against the DL700 material did not recognize any bands unique to ε-negative strains by Western immunoblot. When antiserum made against J48 was adsorbed with DL700 whole cells, the remaining antiserum bound to J48, but not to DL700 whole cells. However, when antiserum made against DL700 gel-purified material was adsorbed with J48 whole cells, antibody binding was decreased against both strains

(data not shown). These results indicate that determinants are recognized by the anti-J48 reagent on ε-positive GBS, but the converse is not true. That is, the anti-DL700 reagent does not recognize additional determinants present on δε-negative, but not on δε-positive, serotype III strains.

Initial characterization of the δ and ε antigens suggests that δ is a protein and that it is associated with one of the three know GBS ACPs. It is not yet known whether this association is specific for the δ antigen or a more general phenomenon involving an interaction of the ACP with multiple GBS proteins. Initial work to isolate and characterize the ε antigen suggests it is a glycoprotein rich in glycine or serine and that it may contain sugars that in part are known to contribute to the group B carbohydrate in addition to sugars not reported to be related to the group B-specific antigen.

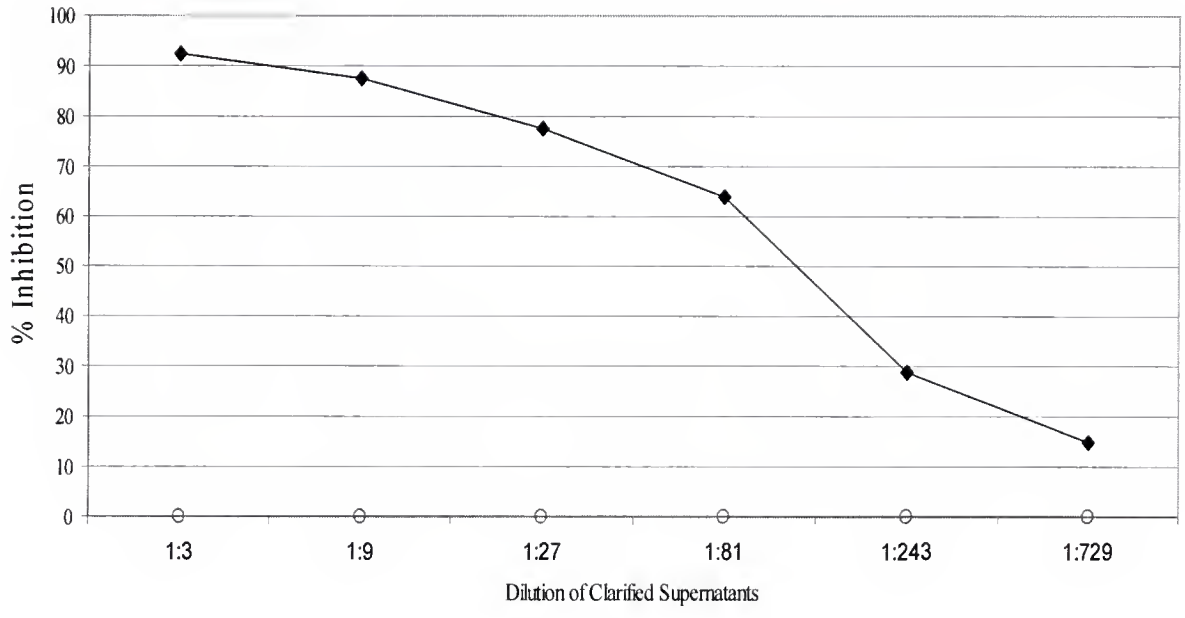
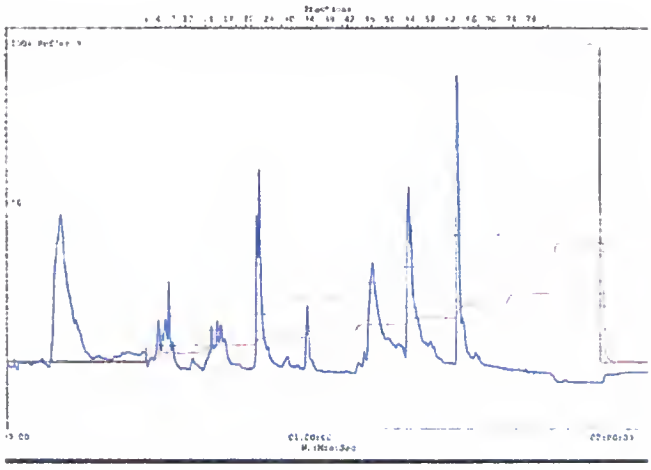



Figure 2-1. Detection of δ/ε in sonic extracts of group B streptococcal (GBS) whole cells by competition ELISA. Sonic extracts of J48 (serotype III/δε) (closed diamonds and DL700 (III) (open circles) were used to inhibit binding of anti-δε antiserum to J48 (serotype III/δε) whole cells. Results are given as % inhibition of anti-δε antibody binding.

A.



B.

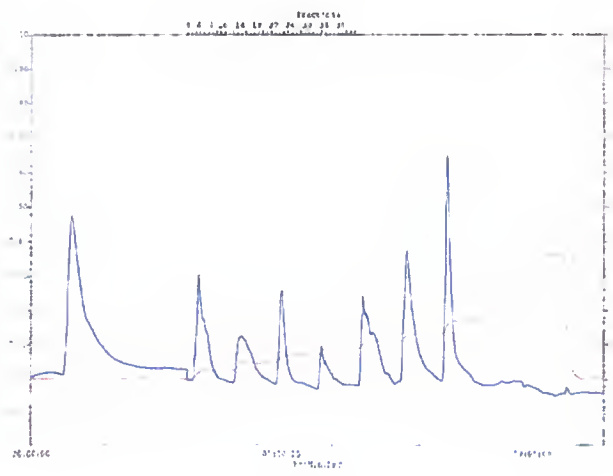
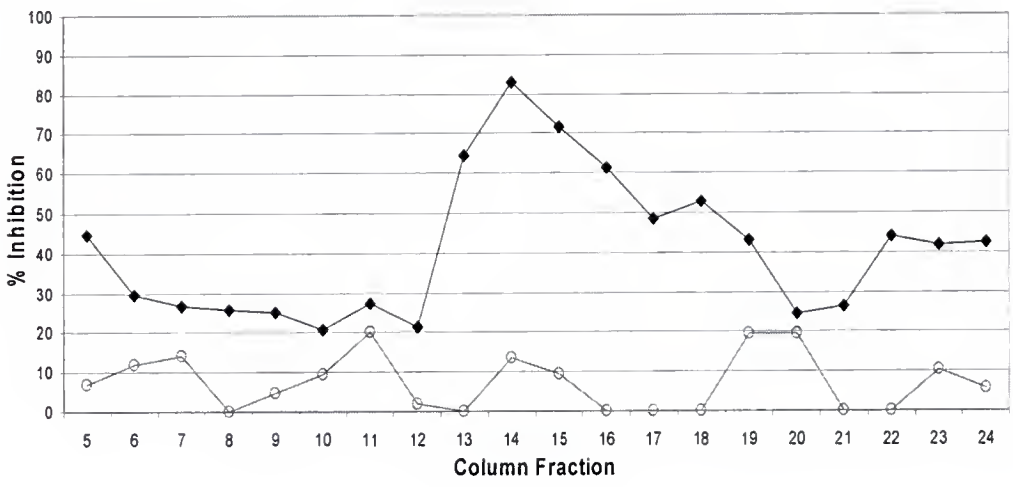


Figure 2-2. Anion-exchange chromatography of GBS sonic extracts. A) Elution profile of J48 (serotype III/δε) sonic extract. B) Elution profile of DL700 (III) sonic extract. $OD_{280}$ is indicated on the y-axis and fraction number on the x-axis. The blue lines represent relative levels of eluted protein. The red lines indicate the concentration of NaCl.

A.




B.

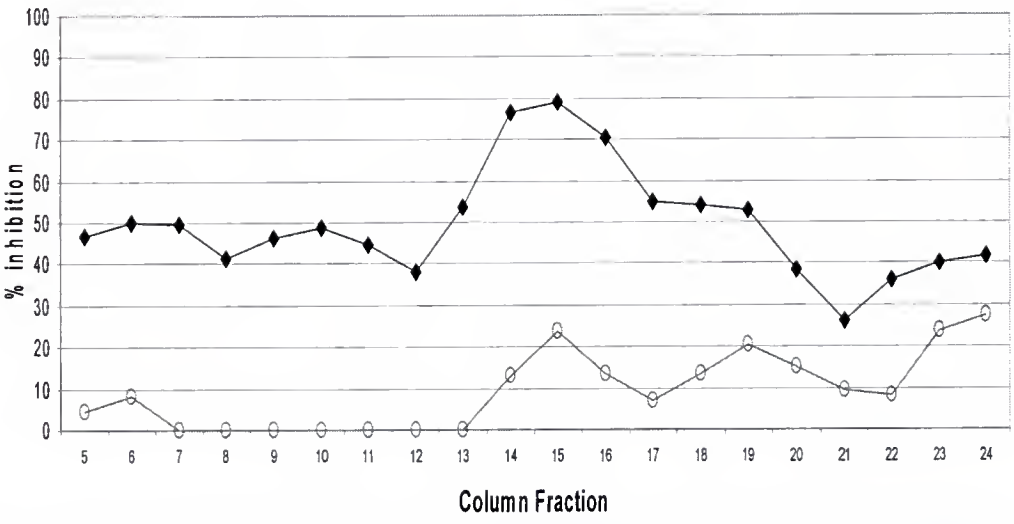



Figure 2-3. Detection of δ and ε in anion-exchange column fractions by competition ELISA. A) Inhibition of anti-δε antiserum binding to A909 (serotype Ic/αβγδ) whole cells B) Inhibition of anti- ε antiserum binding to J48 (serotype III/δε) whole cells. Closed diamonds (♦) represent inhibition by J48 (III/δε) column fractions. Open circles (○) represent inhibition by DL700 (III) column fractions. Results are given as % inhibition as compared to inhibition in the presence of buffer only.

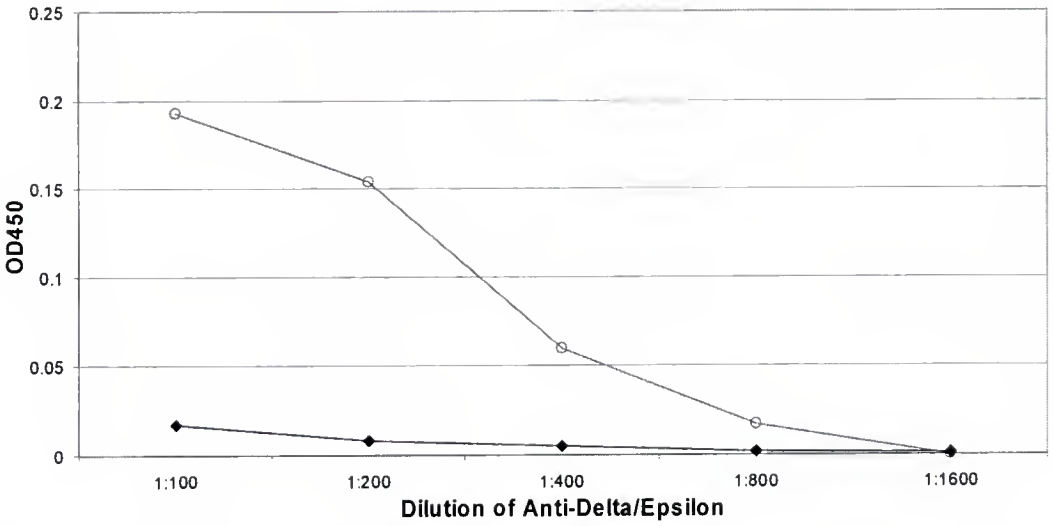



Figure 2-4. Evaluation of proteinase K sensitivity of the δ antigen on A909 (serotype Ic/αβγδ) whole cells by ELISA. A909 whole cells were treated with either proteinase K or buffer only and incubated with anti-δε antiserum. Closed diamonds (♦) represent binding of anti-δε antiserum to proteinase K-treated A909 whole cells, while open circles (○) represent binding of anti-δε antiserum to A909 whole cells treated with buffer only.

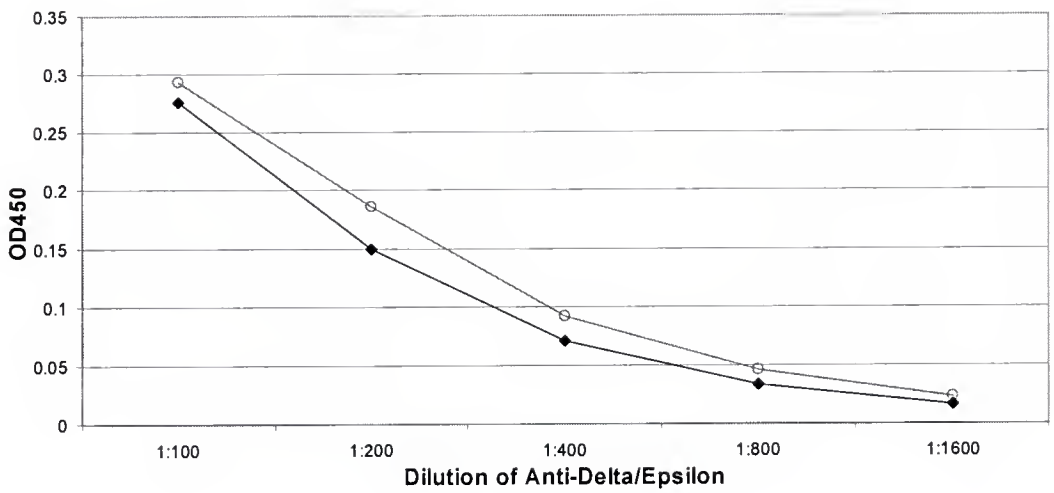



Figure 2-5. Evaluation of sodium metaperiodate sensitivity of the δ antigen on A909 (serotype Ic/αβγδ) whole cells by ELISA. A909 whole cells were treated with either sodium metaperiodate or buffer only and incubated with anti-δε antiserum. Closed diamonds (♦) represent binding of anti-δ antiserum to sodium metaperiodate-treated A909 whole cells, while open circles (○) represent binding of anti-δε antiserum to A909 whole cells treated with buffer only.

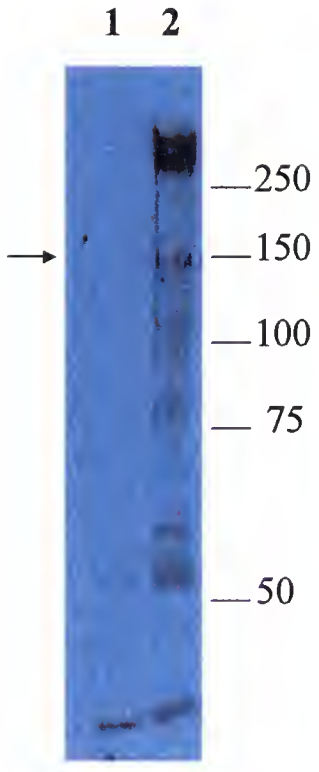



Figure 2-6. Evaluation of anti-δε antiserum reactivity with material contained in anion-exchange column fractions from DL700 (serotype III) and J48 (serotype III/δε). Anion-exchange column fractions #14 from DL700 (Lane 1) and J48 (Lane 2) were separated on a 7.5% SDS-polyacrylamide gel and electroblotted onto nitrocellulose, and incubated with anti-δε antiserum enriched for anti-δ antibodies by immunoprecipitation with strain A909 (serotype Ic/αβγδ).

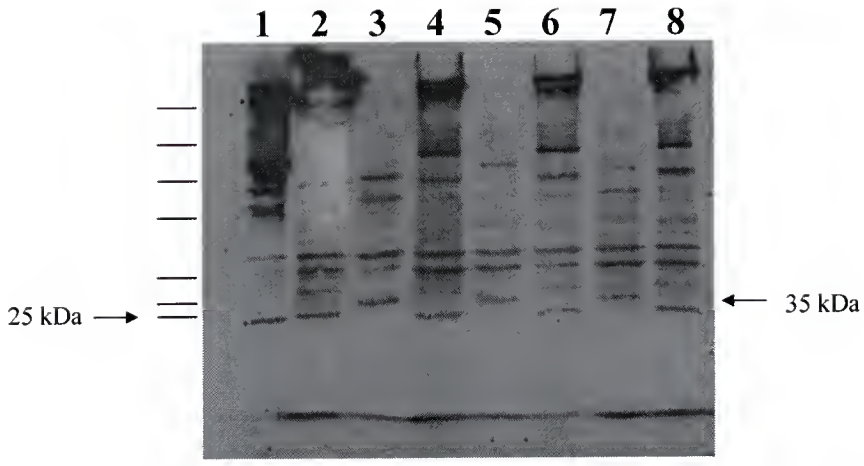



Figure 2-7. Evaluation of anti-δε antiserum reactivity with sodium-dodecyl sulfate (SDS)-extracted material from representative δε-surface positive and δε-surface negative GBS strains. Whole bacterial cells were boiled in SDS sample buffer and extracted material was separated on a 7.5% SDS-polyacrylamide gel, electroblotted onto nitrocellulose, and incubated with anti-δε polyclonal rabbit antiserum enriched for anti-δ antibodies by immunoprecipitation with strain A909 (serotype Ic/αβγδ). Lanes: 1, A909, 2, COH1 (serotype III/δε), 3, ATCC 12403 (serotype III), 4, PEH (serotype III/δε), 5, DL1104 (serotype III), 6, J52 (serotype III/δε), 7, DL700 (serotype III), 8, J48 (serotype III/δε). Molecular mass markers in kDa from top are: 250, 150, 100, 75, 50, 37, and 25.

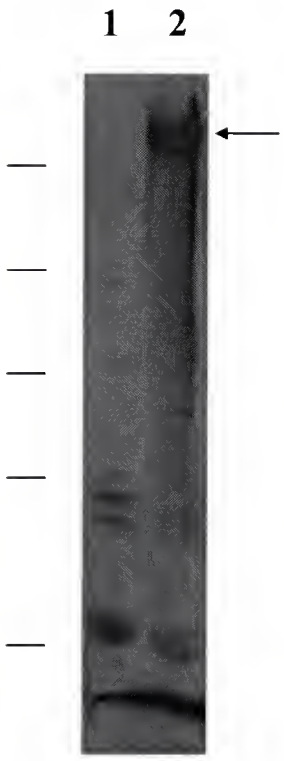



Figure 2-8. Visualization of ε by Western immunoblot. Materials contained within anion-exchange column fractions #14 of DL700 (serotype III) (Lane 1) and J48 (serotype III/δε) (Lane 2) were separated on a 7.5% SDS-polyacrylamide gel, electroblotted onto nitrocellulose, and incubated with anti-ε antiserum. A high molecular weight band (>250 kDa) present in the J48 (serotype III/δε) column fraction was identified as the ε antigen (arrow). Molecular mass markers in kDa from top are: 250, 150, 100, 75, and 50.

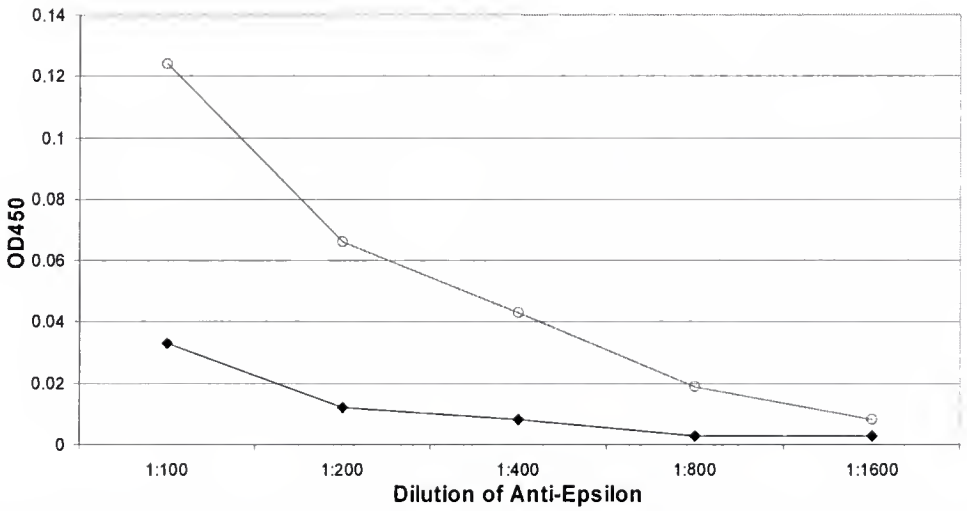



Figure 2-9. Evaluation of proteinase K sensitivity of the ε antigen on J48 whole cells by ELISA. Closed diamonds (♦) represent binding of anti-ε antiserum to proteinase K-treated J48 (serotype III/δε) whole cells, while open circles (○) represent binding of anti-ε antiserum to J48 whole cells treated with buffer only.

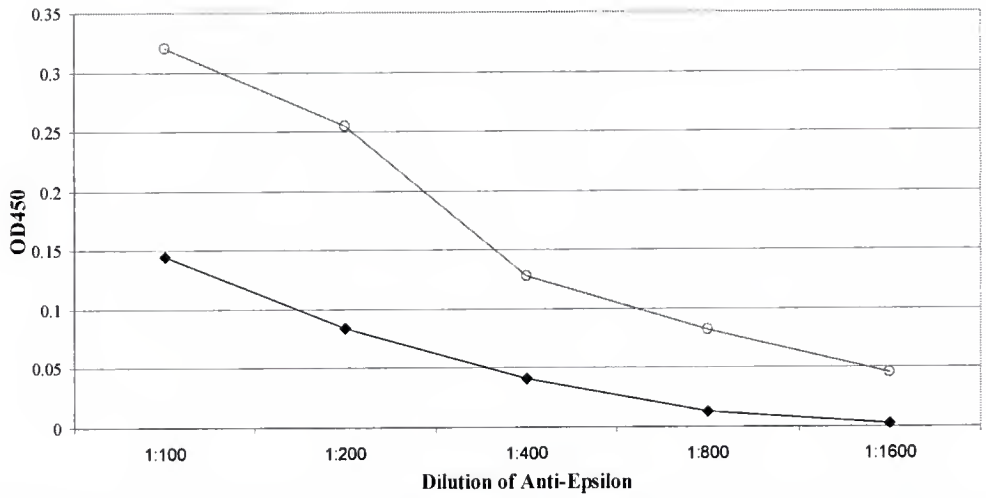



Figure 2-10. Evaluation of sodium metaperiodate sensitivity of the ε antigen on J48 (serotype III/δε) whole cells by ELISA. Closed diamonds (♦) represent binding of anti-ε antiserum to sodium metaperiodate-treated J48 whole cells, while open circles (○) represent binding of anti-ε antiserum to J48 whole cells treated with buffer only.

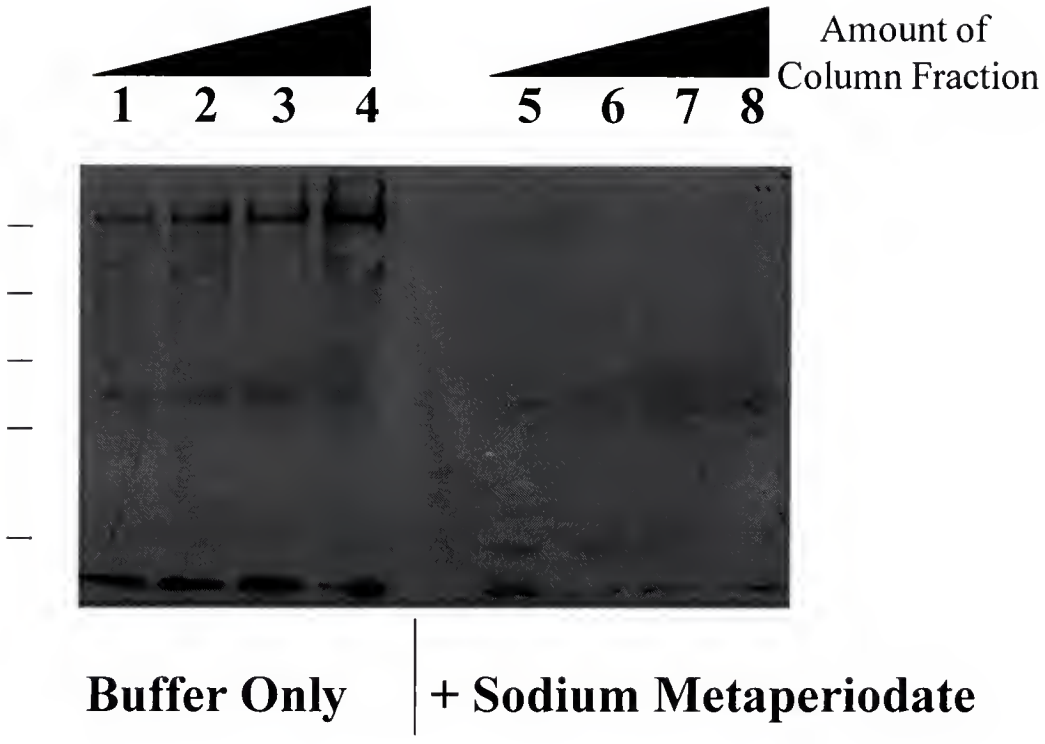



Figure 2-11. Evaluation of sodium metaperiodate sensitivity of the ε antigen assessed by Western immunoblot. Anion-exchange column fraction #14 from GBS strain J48 (serotype III/δε) containing ε was separated on a 7.5% SDS-polyacrylamide gel, electroblotted onto duplicate nitrocellulose membranes, and treated with either buffer or sodium metaperiodate as indicated. Membranes were incubated with anti-ε antiserum. Molecular mass markers in kDa from top are: 250, 150, 100, 75, and 50.

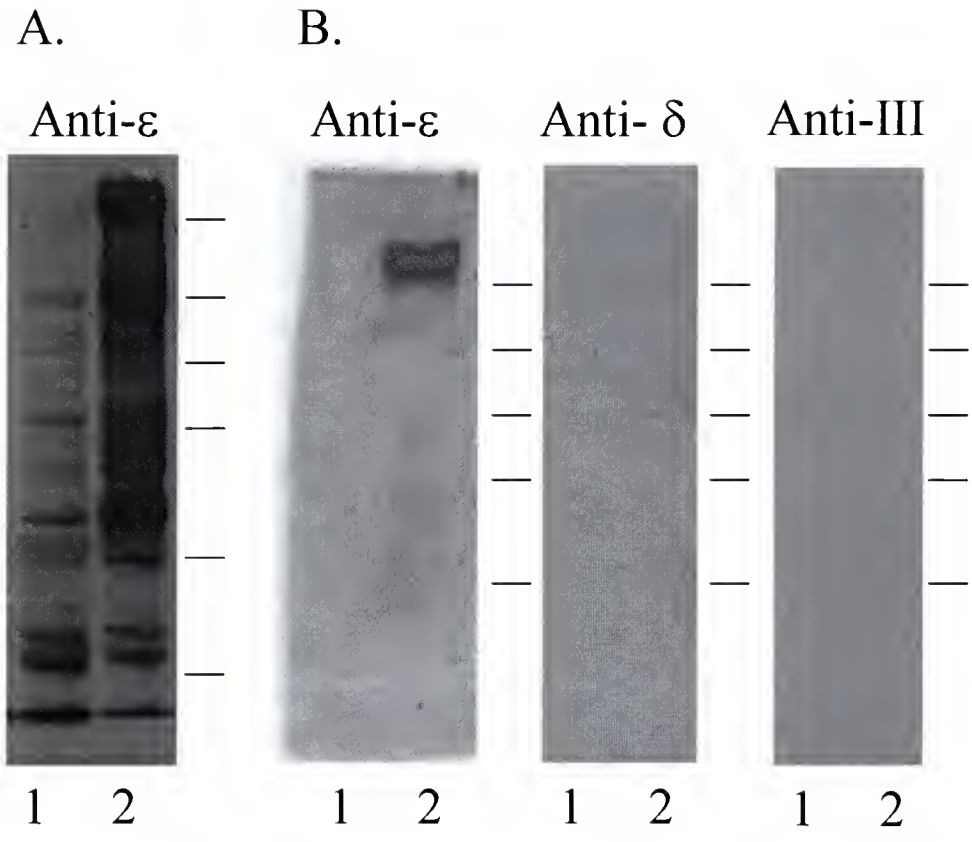



Figure 2-12. Western immunoblot analysis of high-molecular weight material gel-purified from sonic extracts of DL700 (lane 1) and J48 (lane 2). A) Starting material incubated with anti-ε antiserum. B) Gel-purified material incubated with anti-ε, anti-δ, or anti-serotype III antisera. Molecular mass markers in kDa from top are: 250, 150, 100, 75, and 50.

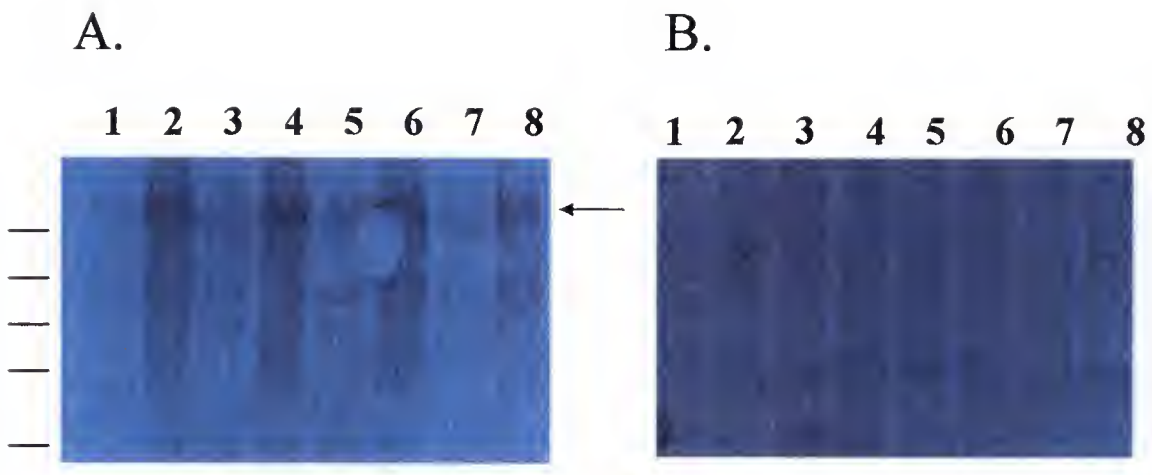



Figure 2-13. Western immunoblot analysis of SDS-extracted material from group B streptococcal (GBS) whole cells. A) Reactivity with polyclonal rabbit antiserum made against ε-containing material from strain J48 (serotype III/δε). B) Reactivity with polyclonal rabbit antiserum made against corresponding material from DL700 (serotype III). Lanes: 1, A909 (serotype Ic/αβγδ), 2, COH1 (serotype III/δε), 3, ATCC 12403 (serotype III), 4, PEH (serotype III/δε), 5, DL1104 (serotype III), 6, J52 (serotype III/δε), 7, DL700 (serotype III), 8, J48 (serotype III/δε). Arrow indicates binding of antiserum from J48 to the >250-kDa ε antigen. Molecular mass markers in kDa from top are: 250, 150, 100, 75, and 50.

# CHAPTER 3
# EVALUATION OF GENETIC COMPONENTS INVOLVED IN δ AND ε EXPRESSION

## Introduction

The δ and ε were initially characterized based on antibody binding. Therefore, the genetic basis for these immunologic reactivities was of interest. Initial work to isolate and characterize the δ antigen by SDS-PAGE and Western immunoblot suggested that δ was associated with altered migration of one of the three acyl-carrier proteins (ACP) shown to be encoded in the genome of GBS (Glaser et al. 2002). In *E. coli*, ACP is involved in carrying substrates to six separate enzymes for sequential processing during fatty acid biosynthesis (Wakil et al. 1983). It has also been shown to be involved in membrane-derived oligosaccharide synthesis (Tang et al. 1997, Therisod et al. 1986) and lipid biosynthesis (Rock and Jackowski 1982). ACP can also interact with proteins in addition to those necessary for fatty acid biosynthesis (Gully et al. 2003). In an attempt to whether ACP represented a component of the δ antigen itself, the gene encoding the GBS ACP associated with anti-δ antibody reactivity was cloned and expressed in *E. coli*. The purified recombinant product was tested by Western immunoblot for reactivity with anti-δ specific antiserum.

In addition to the division of serotype III GBS into two groups based on the co-expression or absence of the δ and ε antigens, serotype III GBS are also categorized into 4 distinct lineages based on *Hind*III and *Sse*83871 restriction digest patterns (RDPs) of chromosomal DNA (Takahashi et al. 1998). The phenotypic consequences of these

genetic differences are not yet understood. In collaboration with Dr. John Bohnsack and Dr. Elisabeth Adderson, the relationship between RDP type and ε antigen expression was explored.

As described in the preceding chapter, the ε antigen was putatively identified as a >250-kDa glycoprotein. Amino acid analysis of ε-containing material suggested the presence of a protein rich in either glycine or serine. A search of the available GBS genomic databases (Glaser et al. 2002, Tettelin et al. 2002) revealed no ORFs encoding a glycine-rich protein. However, an ORF encoding a serine-rich protein was identified. The predicted amino acid sequence of this serine-rich protein was that of a ~125-kDa protein with a repetitive di-serine (XS) motif comprising approximately two-thirds of the protein. Serine-rich repeat (Srr) proteins have recently been described in *Streptococcus gordonii* (Bensing and Sullam 2002, Takahashi et al. 2002) and *Streptococcus parasanguis* (Wu et al. 1998, 1999).

High molecular weight glycoproteins with a highly repetitive di-serine (XS) motif backbone such as Fap1 of *S. parasanguis* (Wu et al. 1999) and GspB (Bensing and Sullam 2002) and Hsa (Takahashi et al. 2002) of *S gordonii* have been reported for several streptococcal species and in the case of GspB are encoded within a locus containing a dedicated accessory secretory apparatus (Bensing and Sullam 2002). These glycoproteins have been characterized as adhesins. Fap1 from *S. parasanguis* binds to saliva-coated hydroxyapatite (Wu et al. 1998, 1999), while *S. gordonii* GspB mediates binding to human platelets (Bensing and Sullam 2002). A similar protein found in another strain of *S. gordonii*, Hsa binds to sialic acid (Takahashi et al. 2002). Although the members of the newly recognized Srr protein family share an XS-motif throughout

the majority of the protein backbone, binding to a given substrate is specific for each individual protein within this family.

A possible relationship between ε antigen expression and a GBS Srr protein was explored. Experiments were conducted in an attempt to identify genetic components involved in, or responsible for, ε expression.

## Materials and Methods

### Bacterial Strains and Growth Conditions

Group B streptococcal (GBS) strains used in this study were A909 (serotype Ic/αβγδ), ss617 (serotype Ia), ss618 (serotype Ib), 9B200 (serotype II/αγ), J48 (serotype III/δε), J52 (serotype III/δε), PEH (serotype III/δε), COH1 (serotype III/δε), COH1-13 (serotype III/δε), DL1104 (serotype III), and DL700 (III). Strains A909, ss617, and ss618 were obtained from Richard Facklam at the Centers for Disease Control and Prevention (Atlanta, GA, USA). Strains 9B200, J48, J52, and PEH were clinical isolates from Shands Hospital, University of Florida. Strains COH1 and COH1-13 were generous gifts from Dr. Craig Rubens (Children's Hospital Regional Medical Center, Seattle, WA, USA). COH1-13 is an acapsular mutant of the parent strain COH1 (Rubens et al. 1987, Wessels et al. 1989). Strains DL1104 and DL700 were clinical isolates kindly provided by Dr. Daniel Lim (University of South Florida, Tampa, FL, USA. All streptococci were grown to late exponential phase in Todd-Hewitt Broth (Becton Dickinson, Cockeysville MD) at 37°C without shaking.

### Cloning and Expression of Recombinant Acyl Carrier Protein (ACP) from J48

Chromosomal DNA from J48 (serotype III/δε) was isolated as described by Nagano et al. (Nagano et al. 1991) and further purified on CsCl gradient. DNA encoding

*acp* was amplified by PCR with J48 DNA as template using the forward primer KS46 and the reverse primer KS47 (Table 3-1) as determined from the *gbs0332* sequence of NEM316 (Glaser et al. 2002). DNA was first denatured at 94°C for 1 min. Thirty amplification cycles were performed as follows: 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 1 min of extension at 72°C, followed by one cycle of 5 min at 72°C. The PCR product was analyzed by gel electrophoresis in 0.7% agarose. The amplified product was ligated to the pBAD-TOPO® (Invitrogen, Carlsbad, CA) expression vector and used to transform *E. coli* strain TOP10 according to the manufacturer's protocol. This plasmid was designated pKS90. The pBAD-TOPO® vector contains the araBAD promoter for tightly regulated expression by L-arabinose, a C-terminal V5 epitope tag for detection of an induced protein, and a C-terminal polyhistidine tag for purification. Correct orientation and reading frame with the C-terminal V5 epitope was confirmed by DNA sequence analysis provided by the ICBR using the universal primers supplied with the pBAD-TOPO® kit (Invitrogen).

**Purification of Recombinant Acyl Carrier Protein (ACP) and Western Immunoblot**

Ten milliliters of mid-log phase *E. coli* cells ($OD_{600}$ = 0.5) containing pKS90 was induced with 10-fold decreasing concentrations of in L-arabinose (from 2 to 0.0002%) to express recombinant ACP. Cells were grown for 4 h after induction. Induced cells were harvested by centrifugation and resuspended in 1 mL lysis buffer (50m M $NaH_2PO_4$ pH 8.0, 0.3 M NaCl, 10 mM imidazole). One milligram of lysozyme (Sigma Chemical Co) was added to each tube and placed on ice for 30 min. Cells were disrupted with a Mini Beadbeater-8 (Biospec Products) 6 × 10 s (5 s rest). Residual intact cells and insoluble material were pelleted by centrifugation for 10 min at 10,000 × *g* and the supernatant was

loaded onto a Ni-NTA Spin Kit column (Qiagen, Valencia CA), and centrifuged through the Ni resin. Unbound material was removed from the column with two washes using 50m M $NaH_2PO_4$ pH 8.0, 0.3 M NaCl, 20 mM imidazole. Bound molecules were eluted from the column with 50m M $NaH_2PO_4$ pH 8.0, 0.3 M NaCl, 250 mM imidazole. As controls, uninduced cultures were treated identically. Material eluted from the column was separated on 10-20% SDS-polyacrylamide gradient gels (Bio-Rad), electroblotted onto nitrocellulose membranes (Protran®, Schleicher & Schuell) and blocked with PBS-Tw. One membrane was incubated with anti-V5 monoclonal antibody (Invitrogen) at a 1:5000 dilution overnight at room temperature. After washing, the blot was incubated with HRP-labeled goat-anti-mouse immunoglobulin (Ig, H+L chain) at a 1:1000 dilution (ICN Biomedicals, Inc) for 2 h at room temperature, washed, and developed with 8 mL of 4-chloro-1-naphthol solution (7 mL PBS, 1 mL 4-chloro-1-naphthol [Sigma Chemical Company; 3 mg/mL in methanol], 8 µl of 30% hydrogen peroxide [Fisher Scientific]). The other membrane was incubated with anti-δ polyclonal rabbit antiserum overnight at room temperature. After washing, the blot was incubated with HRP-labeled goat-anti-rabbit Ig at a 1:1000 dilution (ICN Biomedicals, Inc) for 2 h at room temperature, washed, and developed with ECL™ detection reagents (Amersham Life Sciences).

**Polymerase Chain Reaction (PCR) Amplification of *srr* from GBS Chromosomal DNA**

Chromosomal DNA from GBS was isolated as described by Nagano et al. (1991), purified on CsCl gradients, and used as template for PCR amplification of *srr*. DNA encoding *srr* was amplified using primers KS13(F) and KS14(R) (Table 3-1) as determined from the *srr* sequence of NEM316 (*gbs1529*, Glaser et al. 2002). DNA was first denatured at 94°C for 1 min. Thirty amplification cycles were performed as follows:

1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 2 min of extension at 72°C, followed by one cycle of 5 min at 72°C. The PCR products were analyzed by gel electrophoresis in 0.7% agarose.

**Detection of *srr* by Southern Hybridization**

DNA hybridization was performed by the method of Southern (Sambrook 1989). Chromosomal DNA from all GBS strains was isolated as described by Nagano et al. (1991) and further purified on CsCl gradients. Two micrograms of genomic DNA from each of the GBS strains tested was digested with *Eco*RV (Promega, Madison, WI) at 37°C overnight. The digested DNA was separated by electrophoresis through 0.7% agarose gels and transferred to a nylon membrane (Boehringer Mannheim, Indianapolis, IN) by a capillary blot procedure according to the manufacturer's instructions. The membrane was probed with the DL700 (serotype III) *srr* ORF labeled during PCR amplification with Digoxigenin-11-dUTP (DIG-dUTP, Boehringer Mannheim) at 42°C overnight. DNA encoding *srr* from DL700 was amplified using primers KS13(F) and KS14(R) (Table 3-1) as determined from the *srr* sequence of NEM316 (gbs1529, Glaser et al. 2002). DNA was first denatured at 94°C for 1 min. Thirty amplification cycles were performed as follows: 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 2 min of extension at 72°C, followed by one cycle of 5 min at 72°C. The PCR product was analyzed by gel electrophoresis in 0.7% agarose. The blot was developed according to the manufacturer's instructions.

**RNA Dot Blot to Detect *srr* mRNA**

Total RNA was isolated from streptococci using the RNeasy Mini Kit (Qiagen, Valencia CA) according to the manufacturer's instructions. Serial dilutions of total RNA were transferred to a nylon membrane (Boehringer Mannheim) using a dot blot apparatus

(Schleicher & Schuell). The membrane was incubated with the DL700 (serotype III) *srr* ORF labeled with DIG-dUTP (Boehringer Mannheim) during PCR amplification. The blot was developed according to the manufacturer's instructions.

**Polymerase Chain Reaction (PCR) of GBS Genes Within the *secA2-Y2* locus of Sequenced GBS Strain NEM316**

Forward and reverse primers were designed to amplify individual ORFs contained in the *secA2-Y2* locus (Bensing and Sullam 2002) identified within the published NEM316 (serotype III, RDP III-1) genome sequence (Glaser et al. 2002). These primers are listed in Table 3-1. Chromosomal DNA from J48 (serotype III/δε) and from DL700 (serotype III) was used as template. DNA was first denatured at 94°C for 1 min. Thirty amplification cycles were performed as follows: 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 2 min of extension at 72°C, followed by one cycle of 5 min at 72°C. The PCR products were analyzed by gel electrophoresis in 1% agarose.

**Evaluation of ε Expression by GBS Strains of RDP III Types 1-4**

Costar High Binding plates (Costar) were used. Sample wells were coated overnight at 4°C in a moist chamber with 100 µl of approximately $10^7$ streptococcal whole cells in 0.1M carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide. The plates were blocked with PBS-Tw. Test wells were incubated with 100 µl of anti-ε antiserum (1:100 dilution in PBS) for 2 h at 37°C. After washing 3 times with 300 µl PBS-Tw, 100 µl of affinity-purified HRP-labeled goat-anti-rabbit secondary antibody (1:1000 dilution in PBS) (ICN Biomedicals, Inc) was added to the wells for 2 h at 37°C. Plates were washed, and developed with o-phenylenediamine in 0.1M citric acid/0.2M sodium phosphate buffer in the presence of 0.03% hydrogen peroxide. $OD_{450}$ of test wells was determined using a Model 550 microplate reader (Bio-Rad). Control wells included

incubation of streptococcal whole cells with secondary antibody alone. A strain was designated positive for ε expression if the $OD_{450}$ value observed was more than twice that of the negative control strain DL700 (serotype III, RDP III-2).

**Detection of RDP III-3 Variant *srr* by Southern Hybridization**

DNA hybridization was performed by the method of Southern (Sambrook 1989). Chromosomal DNA from all GBS strains was isolated as described by Nagano et al. (1991) and further purified on CsCl gradients. Two micrograms of genomic DNA from each of the GBS strains tested was digested with *Eco*RV (Promega, Madison, WI) at 37°C overnight. The digested DNA was separated by electrophoresis through 0.7% agarose gels and transferred to a nylon membrane (Boehringer Mannheim, Indianapolis, IN) by a capillary blot procedure according to the manufacturer's instructions. DNA encoding *srr* from GBS strain J48 (serotype III/δε) was amplified by PCR using primers KS51(F) and KS53(R) based on the sequence of an *srr* gene contained within clone DY-3 derived from strain 874391 (RDP III-3) (Bohnsack et al. 2002). This *srr* was similar to, but different from, that identified in the genome of the sequence strain NEM316 (Glaser et al. 2002). DNA was first denatured at 94°C for 1 min. Thirty amplification cycles were performed as follows: 1 min of denaturation at 94°C, 2 min of annealing at 60°C, and 2 min of extension at 72°C, followed by one cycle of 5 min at 72°C. The PCR product was analyzed by gel electrophoresis in 0.7% agarose. The blot was developed according to the manufacturer's instructions.

**Cloning of *srr* from J48**

The *srr* gene from GBS strain J48 (serotype III/δε) was amplified by PCR as described above. The amplified product was ligated to pBAD-TOPO® (Invitrogen)

expression vector and used to transform *E. coli* strain TOP10 according to the manufacturer's protocol. The resulting plasmid was designated pKS60. Correct orientation and reading frame with the C-terminal V5 epitope was confirmed by DNA sequence analysis provided by the ICBR using the universal primers supplied with the pBAD-TOPO® kit (Invitrogen).

**Reactivity of Anti-ε Antiserum with Recombinant Srr from J48**

Ten milliliters of mid-log phase *E. coli* cells ($OD_{600}$ = 0.5) harboring pKS60 was induced to express recombinant Srr with 10-fold increases in L-arabinose (from 0.0002 to 2%). Cells were grown for 4 h after induction, harvested by centrifugation, and resuspended in 1 mL of 1X SDS-sample buffer. The cells were boiled for 5 min and pelleted. Fifty microliters of the supernatant was separated on 7.5% SDS-polyacrylamide gels, electroblotted onto nitrocellulose membranes (Protran®, Scleicher & Schuell), and blocked with PBS-Tw. One membrane was incubated with anti-V5 monoclonal antibody (Invitrogen) at a 1:5000 dilution overnight at room temperature. After washing, the blot was incubated with HRP-labeled goat-anti-mouse Ig (H+L chain) at a 1:1000 dilution (ICN Biomedicals, Inc, Aurora, OH) for 2 h at room temperature, washed, and developed with 8 mL of 4-chloro-1-naphthol solution (7 mL PBS, 1 mL 4-chloro-1-naphthol [Sigma Chemical Company; 3 mg/mL in methanol], 8 µl of 30% hydrogen peroxide [Fisher Scientific]). The other membrane was incubated with anti-ε antiserum overnight at room temperature. After washing, the blot was incubated with HRP-labeled goat-anti-rabbit Ig at a 1:1000 dilution (ICN Biomedicals, Inc, Aurora, OH) for 2 h at room temperature, washed, and developed with ECL™ detection reagents (Amersham Life Sciences, San Francisco, CA).

## Results

### Western Immunoblot of Recombinant Acyl-Carrier Protein

Because anti-δ antiserum reacted with bands that demonstrated sequence homology to a GBS acyl-carrier protein, the gene encoding the ACP was amplified by PCR, cloned, expressed to determine if ACP represented a component of the δ antigen. The recombinant ACP protein expressed in *E. coli* was purified on nickel resin as described above. Starting material, wash material, and eluted material from the Ni column from induced and uninduced cultures were tested for reactivity with anti-V5 and anti-δ antiserum by Western immunoblot (Figure 3-1). The anti-V5 reagent (panel A) recognized a band of ~12 kDa that was present in the starting material of the uninduced culture (lane 1) and was eluted from the nickel resin column (lane 3). This is the predicted size of the recombinant ACP with V5 epitope and histidine tag. The band was not present in the samples derived from the uninduced culture (lanes 4-6) and was concluded to represent the V5 epitope and histidine-tagged recombinant ACP. A band migrating at ~60 kDa recognized by the anti-V5 antibody and present in both the induced (lane 1) and uninduced (lane 4) starting material was not purified on nickel resin and was considered to be an irrelevant anti-V5 cross-reactive moiety unrelated to ACP. The anti-δ antiserum (panel B) did not react with the ~12 kDa recombinant ACP present in the eluted sample from the induced culture (lane 3) indicating that ACP itself is not the δ antigen. Reactivity of the polyclonal anti-δ antiserum with components present in the starting materials of the induced (lane 1) as well as uninduced (lane 4) cultures represents background reactivity of this reagent with *E. coli* proteins unrelated to expression of recombinant ACP.

**Polymerase Chain Reaction (PCR) Amplification of *srr* from GBS Chromosomal DNA**

Preliminary carbohydrate and amino acid analysis of ε-containing material suggested the presence of a protein component rich in either glycine or serine. Examination of the GBS genomic sequence (Glaser et al. 2002) revealed no hypothetical glycine-rich protein, but did identify one open reading frame (ORF) with a deduced amino acid sequence rich in serine. PCR was used to attempt to amplify DNA corresponding to this *srr* (serine rich repeat) gene from multiple GBS strains and amplified products were separated on a 1% agarose gel (Figure 3-2). This *srr* gene was only amplified from δε-negative strains of GBS including δε-negative serotype III strains and serotype Ic, Ib, and II strains. Other serotypes were not tested.

**Southern hybridization with *srr* from DL700**

To determine if the lack of PCR amplification of *srr* observed for serotype III/δε-positive strains was a result of a lack of the gene itself or a consequence of inadequate amplification using primers designed based on the NEM316 sequence (Glaser et al. 2002), Southern analysis was performed (Figure 3-3). The *srr* gene was amplified and labeled from the δε-negative serotype III strain DL700 and used as a probe to detect the presence of the *srr* gene in the same panel of GBS strains used for PCR amplification. Again, no δε-positive strains demonstrated the presence of the *srr* gene; whereas, chromosomal DNA from all δε-negative strains, including serotype III and non-serotype III GBS, hybridized with the *srr* probe derived from DL700.

**Dot Blot to Detect *srr* mRNA**

Because *srr* DNA could not be detected in chromosomal DNA from ε-positive GBS by PCR or Southern hybridization, an RNA dot blot procedure was used to

determine if *srr* might be located on an extra-chromosomal element in those strains.(Figure 3-4). To evaluate the presence of *srr* mRNA, hybridization was conducted using total RNA from J48 (serotype III/δε) and DL700 (serotype III) immobilized on a nylon membrane and incubated with DIG-labeled *srr* PCR product amplified from DL700 chromosomal DNA as the probe (Figure 3-4). RNA from *S. mutans* strain MK4 was included as a negative control. No ORFs encoding serine-rich repeat proteins are present in the *S. mutans* genomic sequence (Ajdic et al. 2002). The probe hybridized with RNA from DL700, but not J48, suggesting that *srr* is not present on an extra-chromosomal element in δε-positive serotype III GBS. The collective results of PCR, Southern hybridization, and RNA dot blot suggested that the *srr* ORF present in the NEM316 database (Glaser et al. 2002) was only present in ε-negative strains, but not ε-positive GBS strains. This information also suggested that serotype III NEM316 would be a δε-negative strain. A whole-cell ELISA assay confirmed this to be the case (data not shown).

**Polymerase Chain Reaction (PCR) Amplification of ORFs Located in the *srr*-Containing Locus**

As stated above, δε-positive serotype III GBS strains did not possess the *srr* ORF present in the NEM316 genome (Glaser et al. 2002). The *srr* ORF is located in a putative operon similar to that of a locus in *S. gordonii* named *secA2-Y2* because of the presence of additional *secA* and *secY* homologues dedicated to the export of GspB, a Srr homologue (Bensing and Sullam 2002). Since δε-positive serotype III GBS strains did not possess the *srr* ORF, it was possible that other ORFs in this locus were missing as well. Therefore, PCR amplification of other ORFs in the locus was conducted using J48 (serotype III/δε) and DL700 (serotype III) chromosomal DNA as template (Figure 3-5).

PCR products were visualized for all ORFs amplified from DL700 chromosomal DNA. The only PCR products visualized using J48 chromosomal DNA were *mutT* and *uvrB*. In the NEM316 genome (Glaser et al. 2002), these genes are approximately 24 kb apart, suggesting that δε-positive GBS lack this 24-kb segment of DNA.

**Evaluation of ε-Expression by RDP III Types 1-4**

In addition to immunological classification of serotype III GBS into two groups based on the presence or absence of the δ and ε antigens, serotype III GBS can be subdivided into four distinct lineages based on *Hind*III and *Sse*83871 restriction digest patterns (RDPs) of chromosomal DNA (Takahashi et al. 1998). In collaboration with Dr. John Bohnsack (University of Utah Health Sciences Center, Salt Lake City, UT) and Dr. Elisabeth Adderson (St. Jude Children's Research Hospital, Memphis TN), representatives of RDP III types 1, 2, 3, and 4 were assayed for ε expression by whole cell ELISA (Table 3-2) to determine if there was a correlation between ε-expression and RDP type. A strain was determined to be positive for ε expression if the ELISA $OD_{450}$ value observed was more than twice that of the negative control strain DL700. The $OD_{450}$ values observed for RDP III-3 strains varied from a high value of 0.45 to a low of 0.13. The $OD_{450}$ value observed for the negative control strain DL700 (serotype III, RDP III-2) was 0.06. Using this criterion, no RDP 1, 2, or 4 strain was positive for ε expression, while all fifteen RDP III-3 strains tested were ε-positive. Subsequent molecular typing by Dr. Bohnsack's lab of three serotype III/δε-positive strains (J48, J52, and PEH) and two serotype III/δε-negative strains (DL700, DL1104) from our laboratory collection revealed that the former strains were RDP III-3 strains, while the latter strains were non-III-3 (data not shown). The majority (91%) of serotype III invasive neonatal isolates have been

reported to be RDP III-3 (Takahashi et al. 1998). Brady et al. (1996) also found that serotype III GBS isolate from septic sources appeared to exhibit a higher frequency of expression of δε (83.3%) compared to serotype III isolates from colonizing sources (58.8), although the sample size was to small to ascertain statistical significance. Taken together, these results indicate that δ/ε expression by serotype III GBS is an antigenic marker for the highly pathogenic RDP III-3 lineage.

**Variant *srr* Associated with RDP III-3 δε-Positive GBS**

Bohnsack et al. (2002) used genomic subtractive hybridization to identify DNA sequences unique to RDP III-3 strains. One clone, designated DY-3, contained DNA encoding a large putative protein rich in serine, with a repetitive di-serine (XS) motif for approximately two-thirds of the protein. This putative protein appeared to represent a variant of the large serine-rich protein found in the published genomic databases of a serotype III and serotype V strain (Glaser et al. 2002, Tettelin et al. 2002). Both variants of *srr* encode an XS motif for approximately two-thirds of the entire protein, however, the DY-3 *srr* is slightly smaller (3.6 kb) than the NEM316 variant (4.0 kb). In addition to primary nucleotide sequence differences, in the NEM316 variant, the amino acids that alternate with serine in the XS motif are alanine, threonine, and methionine, while the DY-3 variant has alternating amino acids glutamic acid, isoleucine, valine, and serine. There are also significant amino acid differences between the N-terminal small XS repeat (~80 amino acids) and the large C-terminal XS repeat (~800 amino acids).Therefore, GBS strains were tested by Southern hybridization to determine the relationship between δ/ε expression by serotype III GBS and the presence of the serine-rich repeat (*srr*) ORF contained within the DY-3 sequence (Bohnsack et al. 2002) (Figure 3-6). The

DIG-labeled *srr* PCR product amplified from J48 (serotype III/δε) chromosomal DNA using primers based on the DY-3 sequence hybridized with all of the serotype III/δε-positive strains tested, but not with any of the ε-negative strains including A909 (serotype Ic/αβγδ). This suggests that RDP III-3, ε-positive strains have a variant *srr* gene, which is similar to, but different from, the *srr* gene contained within the NEM316 genome (Glaser et al. 2002).

**Recognition of a Recombinant Srr by Anti-ε Antiserum**

Reactivity of anti-ε antiserum with GBS whole cells is sensitive to both proteinase K and sodium metaperiodate, suggesting that the ε antigen is comprised of both carbohydrate and protein. In order to determine if the protein encoded by *srr* contained in clone DY-3 represents a protein component of the ε antigen, the *srr* ORF amplified by PCR from J48 (serotype III/δε) was cloned into pBAD-TOPO® (Invitrogen) expression vector and induced to express the Srr protein. Cell lysates were assayed by Western immunoblot for the presence of the recombinant protein using an anti-V5 monoclonal antibody (Figure 3-7, Panel A). A reactive band was detected at >250 kDa. The expressed recombinant Srr was also reactive with anti-ε antiserum by Western immunoblot (Figure 3-7, Panel B). The apparent molecular mass of >250 kDa is bigger than the size predicted for the recombinant Srr (125 kDa); however, when the protein was analyzed under strongly denaturing conditions in the presence of 9 M urea, migration of Srr was as predicted (data not shown). This suggests that Srr may have migrated as a dimer when urea was absent from the gel. The reactivity of recombinant Srr with anti-ε antibodies suggests that the Srr protein from J48 is a component of the ε antigen. Reactivity of anti-ε antibodies with J48 whole cells was diminished but not completely destroyed by

treatment of whole cells with sodium metaperiodate. Treatment of whole cells with proteinase K virtually eliminated anti-ε reactivity (see Chapter 2). These results are consistent with Srr comprising the protein backbone of a glycoprotein corresponding to the ε antigen.

**Schematic Representations of Variant *srr*-Containing Loci**

The *srr*-containing locus identified in the NEM316 genome sequence (Glaser et al. 2002) has both *secA2* and *secY2* homologues as well as six ORFs encoding putative glycosyltransferases and several ORFs encoding hypothetical proteins of unknown function (Figure 3-8, panel A). In this organization, the *srr* and *secY2* genes are separated by approximately 12-kb of DNA that includes the glycosyltransferases genes. In the variant locus associated with serotype III/δε-positive RDP III-3 GBS (Panel B), the *secY2* gene lies immediately adjacent (113 nt) and downstream of the *srr* gene. The presence of glycosyltransferases genes or homologues of other ORFs contained in the non-RDP III-3 locus is unknown at present as the DY-3 clone does not include DNA downstream of *secY2*. It is interesting that insertion sequences for the Tn10 transposon are contained within the DY-3 clone. The proximity of such insertion sequences to the *srr*-containing variant locus associated with RDP III-3 strains suggests a genetic recombination event in this lineage.

## Discussion

Initial work to isolate and characterize the δ antigen by SDS-PAGE and Western immunoblot suggested that δ is associated with one of the three known GBS acyl-carrier proteins (ACP) identified in the NEM316 (Glaser et al. 2002) genomic sequence. One of the δ-reactive bands demonstrating an N-terminal sequence corresponding to ACP and

associated with A909 (Ic/αβγδ) and serotype III/δε-positive GBS had an apparent molecular mass of ~25 kDa. Curiously, serotype III/δε-negative GBS lacked this band but demonstrated an anti-δ reactive band migrating at ~35 kDa, also with N-terminal sequence homology to the same ACP. The ACP has a predicted size of ~10 kDa; therefore, these bands likely represent ACP complexed with other moieties. It is unclear why anti-δ antibodies react with the ~35 kDa band from serotype III/δε-surface negative strains. It is possible that a variant form of δ is expressed by these strains but that δ epitopes are not exposed on the cell surface. It is also possible that the ACP-associated moiety detected in these strains is unrelated to δ. As stated previously, the anti-δ antiserum recognizes δ specifically on whole cells, but reacts with numerous additional antigens in cellular extracts.

ACP is involved in fatty acid (Wakil et al. 1983), oligosaccharide (Tang et al. 1997, Therisod et al. 1886), and lipid biosynthesis (Rock and Jackowski 1982). ACP can also form a disulfide bond with a protein not involved in fatty acid biosynthesis (Gully et al. 2003). To determine whether ACP was the δ antigen itself, the gene encoding the GBS ACP was cloned and the recombinant protein expressed in *E.coli*. Anti-δ antiserum did not bind to the purified recombinant ACP (Figure 3-1). Follow-up studies beyond the scope of this project will be necessary to clarify the relationship between the GBS δ antigen and ACP.

The ε antigen was found to correspond to a high molecular weight antigen (>250-kDa) with both protein and carbohydrate components. Amino acid analysis of ε-containing material suggested the presence of a protein rich in either glycine or serine. By searching GBS genomic databases (Glaser et al. 2002, Tettelin et al. 2002), no glycine-

rich proteins were identified, while an ORF with a deduced amino acid sequence of a serine-rich protein was identified. The deduced amino acid sequence of this serine-rich protein suggested an ~125 kDa protein with a di-serine (XS) motif comprising approximately two-thirds of the protein. PCR amplification of this ORF was obtained using chromosomal DNA only from δε-negative GBS strains as template (Figure 3-2). These strains included multiple serotypes. PCR amplification was not obtained for serotype III/δε-positive strains.

It was possible that the *srr* ORF from δε-positive strains was not amplified due to a sequence variation from the designed primers. Therefore, Southern blot was performed to determine if *srr* was present in δε-positive strains (Figure 3-3). Hybridization with the PCR-amplified *srr* from DL700 (serotype III) was only detected for ε-negative strains, suggesting that the *srr* sequence did not exist in the chromosome of δε-positive strains, or that there were significant differences in the *srr* sequences.

To exclude the possibility that Srr was encoded on an extra-chromosomal element in δε-positive strains, an RNA dot blot experiment was performed (Figure 3-4). The only hybridization observed with the labeled *srr* PCR product from DL700 was with mRNA from that strain. Strain J48 did not demonstrate the presence of *srr* mRNA. This inverse relationship between ε-expression and the presence of the *srr* ORF suggested that the sequenced NEM316 serotype III strain should be δε-negative. A whole-cell ELISA proved that this was indeed the case (data not shown).

The *srr* ORF from the NEM316 genome (Glaser et al. 2002) is located in a locus with significant similarity to a locus in *S. gordonii* described by Bensing and Sullam (2002). In this organism, the Srr protein (GspB) is responsible for binding to human

platelets. The gene encoding GspB is located upstream of *secA* and *secY* homologues, designated *secA2* and *secY2*. The entire locus has been called the *secA2-Y2* locus. Because ε-positive GBS did not contain a *srr* ORF as determined by PCR, DNA hybridization, and RNA dot blot, PCR amplification was used to determine if J48 (serotype III/δε) lacked other ORFs in this locus (Figure 3-5). Only two genes, *mutT* and *uvrB* were amplified from the J48 chromosome, while nine additional ORFs within the 24-kb separating *mutT* and *uvrB* in the NEM316 genomic sequence were amplified from the DL700 (serotype III) chromosome. This suggests that a number of genes present in the *srr*-containing *secA2-Y2* locus of ε-negative GBS are lacking in ε-positive strains; however, technical difficulties associated with PCR amplification cannot be excluded as an explanation for these results.

The work by Takahashi et al. (1998) suggested RDP type III-3 represents a more pathogenic lineage of serotype III GBS, and preliminary epidemiological data (Brady et al. 1996) suggested that δ/ε expression was a marker for a more pathogenic subset of serotype III GBS. Therefore, RDP type (1-4) and the presence or absence of the ε antigen on the cell surface of serotype III strains were evaluated in parallel. All ε-positive strains were III-3, while all ε-negative strains were non-III-3. These results indicate that the highly pathogenic RDP III-3 lineage can be identified immunologically on serotype III GBS, namely by the detection of the co-expressed δ and ε antigens.

Bohnsack et al. (2002) used genomic subtractive hybridization to identify DNA sequences unique to RDP III-3 strains. One clone, designated DY-3, contained DNA encoding a large putative protein rich in serine, with a di-serine (XS) motif for approximately two-thirds of the protein. This putative protein was similar, but not

identical, to the homologous large serine-rich protein found in the published genomic databases of a serotype III and a serotype V strain (Glaser et al. 2002, Tettelin et al. 2002). Therefore, a panel of GBS strains was tested by Southern hybridization to determine which, if any, contained the serine-rich repeat (*srr*) ORF identified within the DY-3 clone (Bohnsack et al. 2002) (Figure 3-6). A correlation was observed between ε expression and the DY-3 variant of *srr*. All RDP III-3/δε-positive GBS strains tested contained the DY-3 *srr* variant; whereas all ε-negative GBS contained the NEM316 *srr* variant (Figure 3-3). Although approximately two-thirds of each Srr is composed of a di-serine (XS) motif, there was not any detectable cross-hybridization between the variant genes. There are two major differences in these two variant Srr proteins. In the NEM316 variant the amino acids that alternate with serine in the XS motif are alanine, threonine, and methionine, while the DY-3 variant has alternating amino acids glutamic acid, isoleucine, valine, and serine. There are also significant amino acid differences between the N-terminal small XS repeat (~80 amino acids) and the large C-terminal XS repeat (~800 amino acids).

Based on SDS-PAGE, Western immunoblot, and composition analysis, ε was determined to likely represent a high molecular weight glycoprotein rich in serine. Since ε-positive GBS contained a variant *srr* with similarity to genes encoding high molecular weight di-serine glycoproteins of *S. gordonii* (Bensing and Sullam 2002) and *S. parasanguis* (Wu et al. 1998), DNA encoding Srr from the ε-positive GBS strain J48 (serotype III/δε) was cloned into an expression vector and the recombinant Srr protein was expressed in *E. coli*. Anti-ε antiserum bound to the induced recombinant Srr protein (Figure 3-7). The predicted molecular mass of this Srr is ~125 kDa. It is unknown why

the recombinant protein migrates at ~250 kDa. In the presence of 9 M urea, the recombinant Srr migrates close to its predicted size. It is possible that under standard SDS-PAGE conditions the recombinant Srr protein migrates as a dimer or that the molecule is not sufficiently denatured and its migration is aberrant. Results suggested that the ε antigen is comprised of both protein and carbohydrate components. It is probable then, that only part of ε antigenic reactivity is represented by recombinant Srr. Additional studies will be necessary to identify possible carbohydrate components and linkages that may contribute to ε antigenic reactivity. Of interest is the relationship of the J48 ε-containing material, and the corresponding serine-rich material from the serotype III ε-negative strain DL700, with the group B carbohydrate antigen. Two sugars, rhamnose and galactose, known to be contained within the group B carbohydrate were detected by carbohydrate analysis of the >250-kDa gel-purified material from sonic extracts of these two strains. The materials were reactive with a group B-specific typing reagent suggesting that a component of the group B carbohydrate is associated with the serine-rich protein variants from both ε-negative and ε-positive strains.

A comparison of the NEM316 genome (Glaser et al. 2002) and the DY-3 sequence (Bohnsack et al. 2002) show that although both DNA sequences contain *secY2* homologues located downstream of their respective *srr* ORF, there is approximately 12-kb of DNA encoding putative glycosyltransferases between the two ORFs in non-III-3 strains. Only 113 nt separate *srr* and *secY2* in the DY-3 sequence. In *Staphylococcus aureus* and *Staphylococcus epidermidis, secY2* lies immediately downstream of *srr*, similar to the organization observed in clone DY-3, while NEM316 has an organization more similar to that of other streptococci (Takamatsu et al. 2004). It is unknown at this

time what functions the glycosyltransferases may play in the non-III-3 strains or whether homologues of some or all of the genes encoding these glycosyltransferases are present elsewhere in RDP III/δε-positive strains. In addition to differences in the protein backbone of the two Srr variants, there may be differences in carbohydrate modifications as well, with different sugars, different linkages, or both. Further work focusing on potential differences in carbohydrate modification of RDP III-3 and non-III-3 strains will likely yield additional information regarding the immunologic basis for ε reactivity as well as elucidate differences in cell surface structures that may be associated with differences in pathogenic potential.

Table 3-1. Primers used in this study.

| Primer Name (Forward/Reverse) | Sequence 5'-3' | Gene Amplified | Reference Sequence[1] |
|---|---|---|---|
| KS46 (F) | atggcagtatttgaaaaagtaca | gbs0332 (*acp*) | NEM316 |
| KS47 (R) | ttttacttttcttcaacataagc | gbs0332 (*acp*) | NEM316 |
| KS13 (F) | atgtcccaaaagacttttggc | gbs1529 (*srr*) | NEM316 |
| KS14 (R) | cgtcccaaaagggttgcaccagtca | gbs1529 (*srr*) | NEM316 |
| KS31 (F) | atgtcacgcagtcaaaaagtta | gbs1514 (*mutT*) | NEM316 |
| KS32 (R) | accatcttcaagtcgcttact | gbs1514 (*mutT*) | NEM316 |
| KS39 (F) | tgatagttacactcaatcaagtt | gbs1516 | NEM316 |
| KS40 (R) | ctgagtagagcttgaaacatg | gbs1516 | NEM316 |
| KS41 (F) | aaccatggaattggttgggc | gbs1517 | NEM316 |
| KS42 (R) | aaccatggaattggttgggc | gbs1517 | NEM316 |
| KS33 (F) | atgacagcctttaatagtttattt | gbs1518 (*secA2*) | NEM316 |
| KS34 (R) | aagatctccattctcattgaag | gbs1518 (*secA2*) | NEM316 |
| KS35 (F) | cctggtgtcttgatttctgct | gbs1522 (*secY2*) | NEM316 |
| KS36 (R) | ttctcctcattttcaaatacaga | gbs1522 (*secY2*) | NEM316 |
| KS24 (F) | agctatcgcgttagcggca | gbs1526 | NEM316 |
| KS30 (R) | gccttgaccatgataagttgt | gbs1526 | NEM316 |
| KS26 (F) | ggtgcagatttccaatatcgt | gbs1527 | NEM316 |
| KS27 (R) | tcatctaagaggacctacttc | gbs1527 | NEM316 |
| KS28 (F) | atgcgagtacatattacaagtat | gbs1528 | NEM316 |
| KS29 (R) | atctataaagcatatgcacagc | gbs1528 | NEM316 |
| KS22 (F) | gataccccctcactatcctt | gbs1530 (*rofA*) | NEM316 |
| KS23 (R) | gatattactgatttgagagggt | gbs1530 (*rofA*) | NEM316 |
| KS20 (F) | atagatagaaaagatactaaccg | gbs1531 (*uvrB*) | NEM316 |
| KS21 (R) | tgcagctagctcaaagtccaataat | gbs1531 (*uvrB*) | NEM316 |
| KS51 (F) | tcacgcaaagttcgagttaaaa | *srr* | DY-3 |
| KS53 (R) | cgcagatttagtagctcctaa | *srr* | DY-3 |

[1] NEM316 refers to the published genome sequence by Glaser et al. (Glaser et al. 2002). DY-3 refers to the RDP III-3 genomic clone described by Bohnsack et al. (Bohnsack et al. 2002)

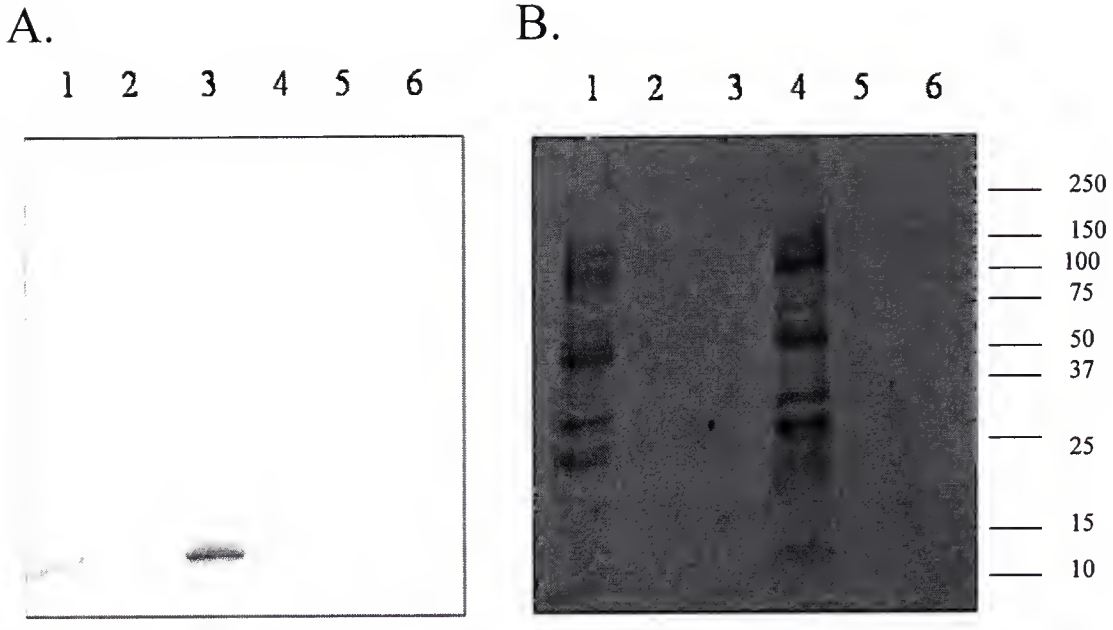



Figure 3-1. Western immunoblot analysis of recombinant GBS acyl-carrier protein. A) Membrane incubated with anti-V5 monoclonal antibodies. B) Membrane incubated with anti-δ antiserum. Lanes: 1, starting material from induced culture, 2, wash from induced culture, 3, elution from induced culture, 4, starting material from uninduced culture, 5, wash from uninduced culture, 6, elution from uninduced culture.

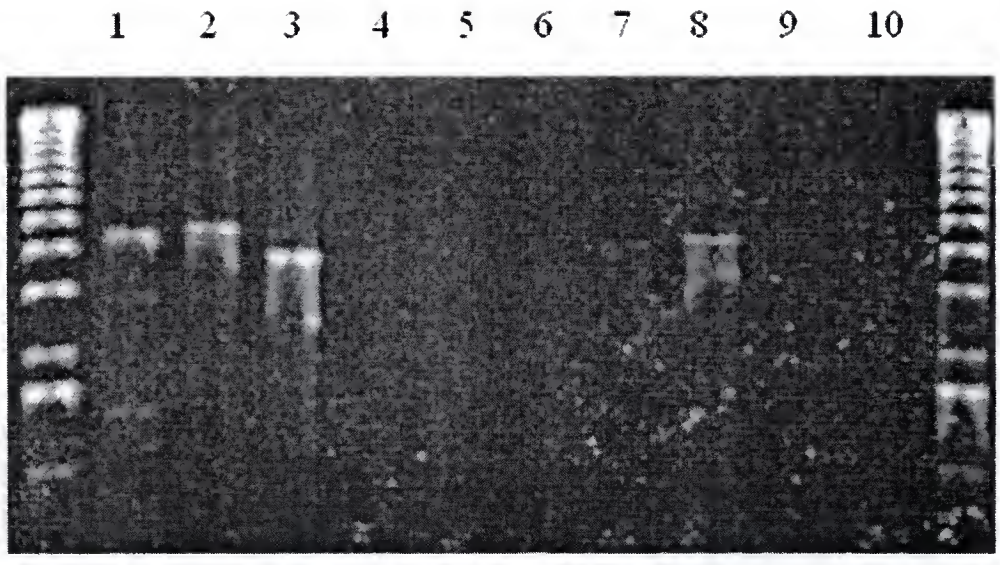



Figure 3-2. Polymerase Chain Reaction (PCR) amplification of *srr* DNA from GBS strains. DNA encoding the *srr* ORF was amplified using primers designed according to the NEM316 genome database. Lanes: 1, A909 (serotype Ic/αβγδ), 2, ss618 (serotype Ib/αβγ), 3, 9B200 (serotype II/αγ), 4, J48 (serotype III/δε), 5, J52 (serotype III/δε), 6, PEH (serotype III/δε), 7, DL700 (serotype III), 8, DL1104 (serotype III), 9, COH1 (serotype III/δε), 10, COH1 (serotype III/δε, acapsular mutant of COH1).

Figure 3-3. Detection of *srr* by Southern hybridization. Hybridization of DL700 (serotype III) *srr* DIG-labeled PCR product with *Eco*RV-digested chromosomal DNA from a panel of GBS strains. Lane 1, A909 (serotype Ic), 2, ss618 (serotype Ib), 3, 9B200 (serotype II/αγ), 4, J48 (serotype III/δε), 5, J52 (serotype III/δε), 6, PEH (serotype III/δε), 7, COH1 (serotype III/δε), 8, COH1-13 (serotype III/δε), 9, DL700 (serotype III), 10, DL1104 (serotype III), 11, pPC185 (negative control), 12, DL700 *srr* PCR product (positive control).

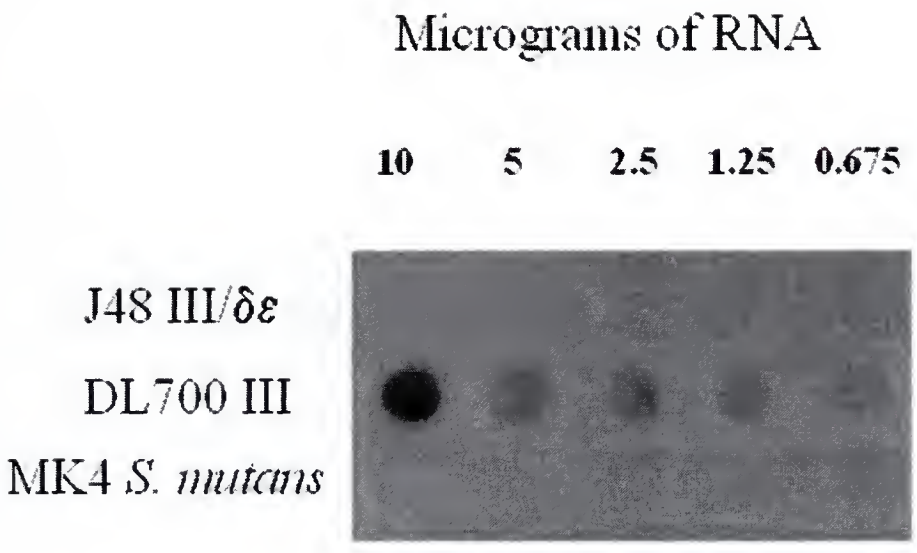



Figure 3-4. Ribonucleic acid (RNA) dot blot to detect *srr* mRNA. Hybridization of RNA from J48 (serotype III/δε), DL700 (serotype III), and S. mutans MK4 negative control with DL700 (serotype III) *srr* DIG-labeled PCR product.

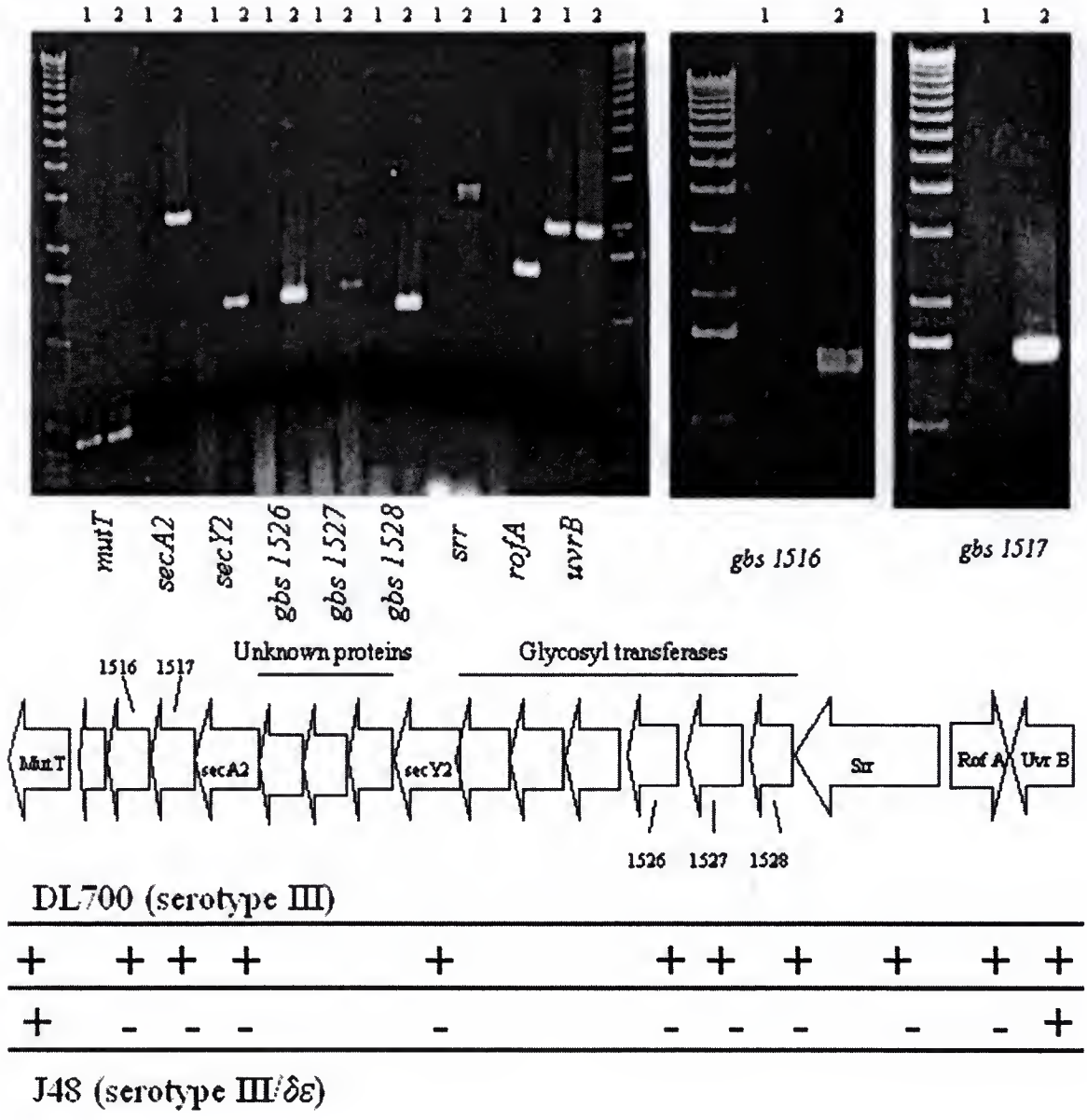



Figure 3-5. Comparison between J48 (serotype III/δε) and DL700 (serotype III) of ORFs contained within an operon including the *srr* variant common to δε-negative GBS strains. Forward and reverse primers to amplify individual ORFs were designed according to the published NEM316 genome sequence (Glaser et al. 2002) and listed in Table 3-1. Chromosomal DNA from J48 (serotype III/δε) and DL700 (serotype III) was used as template. Lanes 1: PCR products amplified from J48 chromosomal DNA, lanes 2: PCR products amplified from DL700 chromosomal DNA. Each ORF tested is labeled. The schematic of the NEM316 loci containing *srr* is shown and PCR results summarized.

Table 3-2. Epsilon expression by group B streptococci (GBS) of RDP types 1-4.

| RDP Type | Number of Strains Tested | Number of Strains Positive for Epsilon Expression | % Positive |
|---|---|---|---|
| III-1 | 2 | 0 | 0 |
| III-2 | 15 | 0 | 0 |
| III-3 | 15 | 15 | 100 |
| III-4 | 3 | 0 | 0 |

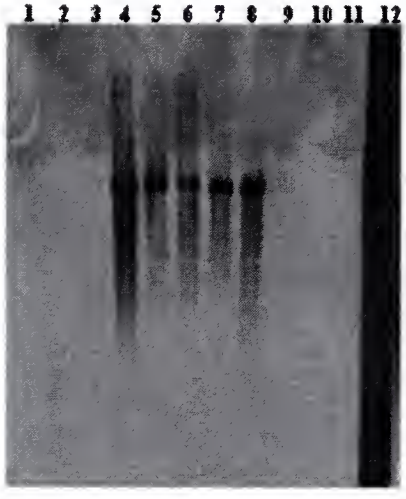



Figure 3-6. Detection of a variant *srr* ORF associated with serotype III/δε GBS by Southern hybridization. Hybridization of J48 (serotype III/δε) *srr* DIG-labeled PCR product (from DY-3 sequence) with *Eco*RV-digested chromosomal DNA. Lane 1, A909 (serotype Ic/αβγδ), 2, ss618 (serotype Ib/αβγ), 3, 9B200 (serotype II/αγ), 4, J48 (serotype III/δε), 5, J52 (serotype III/δε), 6, PEH (serotype III/δε), 7, COH1 (serotype III/δε), 8, COH1-13 (serotype III/δε), 9, DL700 (serotype III), 10, DL1104 (serotype III), 11, pPC185 (negative control), 12, J48 *srr* PCR product (positive control).

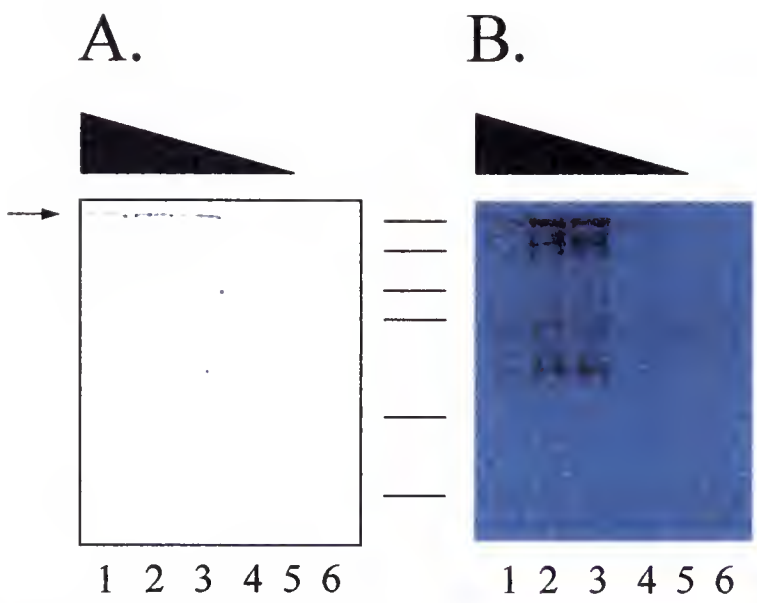
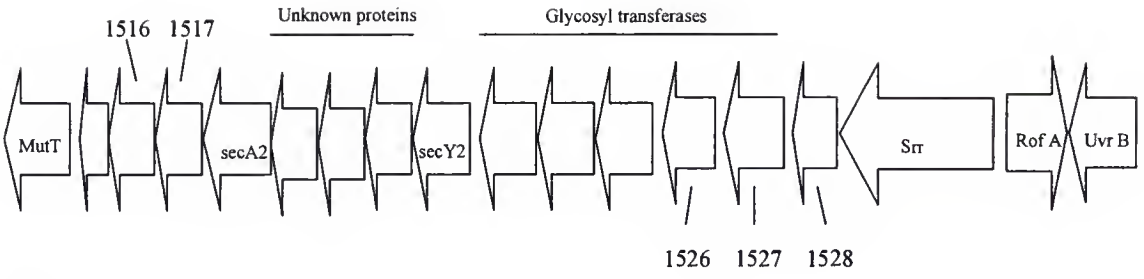



Figure 3-7. Western immunoblot analysis of recombinant Srr from J48 (serotype III/δε). A) Membrane incubated with anti-V5 monoclonal antibodies. B) Membrane incubated with anti-ε polyclonal rabbit antiserum. Lanes 1-5: Cells induced with 10-fold reductions of L-arabinose from 2% (lane 1) to 0.0002% (lane 5). Lane 6: Uninduced control. Arrow denotes induced recombinant Srr as determined by reactivity with anti-V5 monoclonal antibody. Molecular mass markers from top to bottom are 250, 150, 100, 75, 50, and 37 kDa.

A.




B.

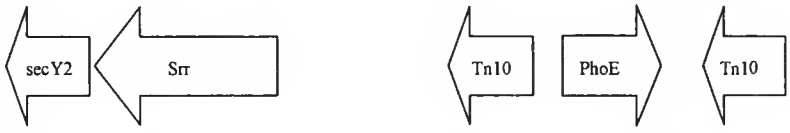



Figure 3-8. Comparison of srr-containing loci representative of non-RDP III-3 and RDP III-3 GBS. A) Schematic of srr-containing locus from the NEM316 genome sequence. B) Schematic of srr-containing locus from the DY-3 sequence.

# CHAPTER 4
# THE ASSOCIATION OF δ AND ε EXPRESSION WITH PATHOGENESIS OF SEROTYPE III GBS

## Introduction

In an initial epidemiological study by Brady et al (1996), the δ and ε antigen were detected on 83.3% of serotype III GBS from septic sources compared to 58.8% of type III isolates from colonized sources. In addition, strain DL700 (serotype III) was readily killed in an opsonophagocytosis assay in the presence of rabbit antiserum made against strain J48 (serotype III/δε); whereas, strain J48 itself was resistant to killing in the presence of the same antiserum. RDP III-3 data generated by Takahashi et al (1998) showed only 33% of vaginal isolates were III-3, while 91% of invasive neonatal isolates were of this RDP type. This paralleled the epidemiological data of Brady et al. (1996), supporting the hypothesis that δ/ε expression by RDP III-3 strains may be markers for a more pathogenic lineage of serotype III GBS. A potential association of δ/ε expression with pathogenesis of serotype III GBS infection was explored using a neonatal mouse model of infection comparing the lethal dose 90% ($LD_{90}$) values of a panel of representative strains.

That neonatal mouse pups can be protected from lethal GBS challenge after administering mothers antiserum made against GBS proteins has been demonstrated. Lancefield et al. (1975) showed protection by rabbit antiserum against unidentified "c-protein" antigens independent of the polysaccharide capsule of GBS. Madoff et al. (1991) showed that monoclonal antibodies recognizing the α antigen are protective in mice. It

has since been shown that antibodies to both the amino-terminal domain and the repeat region of the α protein are protective in an animal model of GBS infection and mediate opsonophagocytic killing in vitro (Kling et al. 1997). Antibodies against the β antigen are also protective in mice (Fusco et al. 1997, Madoff et al. 1992, Michel et al. 1991). Antibodies to protein Rib confer protective immunity (Stalhammar-Carlemalm et al. 1993), as does antiserum recognizing an α-like protein present on serotype V GBS (Lachenauer and Madoff 1996). These studies suggest that antibodies to surface structures in addition to the capsular polysaccharide are important targets of protective immunity. Passive protection studies administering antiserum made against ε-containing material from J48 (serotype III/δε) and corresponding material from DL700 (serotype III) to pregnant mothers, with subsequent challenge of offspring with either strain J48 (serotype III/δε) or DL700 (serotype III) were performed to assess the protective capacity of antibodies directed against ε.

## Material and Methods

### Bacterial Strains and Growth Conditions

Group B streptococcal (GBS) strains used in this study were A909 (serotype Ic/αβγδ), J48 (serotype III/δε), J52 (serotype III/δε), 874391 (serotype III/δε), COH1 (serotype III/δε), DL686 (serotype III), 865043 (serotype III), ATCC12403 (serotype III) and DL700 (serotype III). Strain A909 was obtained from Dr. Richard Facklam at the Centers for Disease Control and Prevention (Atlanta, GA, USA). J48 and J52 were clinical isolates from Shands Hospital. DL686 and DL700 were clinical isolates kindly provided by Dr. Daniel Lim (University of South Florida, Tampa, FL, USA. Strain COH1 was a generous gift from Dr. Craig Rubens (Children's Hospital Regional Medical

Center, Seattle, WA, USA). Strain 874391 (serotype III/δε, RDP III-3) and 865043 (serotype III, RDP III-2) were generous gifts from Dr. Elisabeth Adderson, (St. Jude Children's Research Hospital, Memphis, TN). ATCC12403 (serotype III, RDP III-1) was obtained from American Type Culture Collection (Manassas, VA). All streptococci were grown to late exponential phase in Todd-Hewitt Broth (Becton Dickinson, Cockeysville MD) at 37°C without shaking.

**Evaluation of Lethal Dose 90% of δε-Positive and δε-Negative Serotype III GBS**

A neonatal mouse model of infection was used to compare the lethal dose 90% ($LD_{90}$) values of representative serotype III/δε-positive and serotype III/δε-negative strains. All offspring from a given mother were challenged intraperitoneally with the challenge dose (from $10^2$-$10^8$ cfu) diluted in 100 μl of THB 24-48 h after birth. Deaths were recorded for 72 h post-inoculation. $LD_{90}$ values were determined by the method of Reed-Muench (1938).

**Passive protection assays**

Passive protection assays were conducted based on the mouse neonatal model of infection adapted from Rodewald et al. (1992). Timed-pregnant CD-1 mice (Charles River Laboratories) were injected on day 16 in the tail vein with 500 μl of polyclonal rabbit antiserum made against ε-containing material from GBS strain J48 (serotype III/δε) or 500 μl of antiserum made against the corresponding material from DL700 (serotype III). The generation of these antisera is described in Chapter 2. Preimmune sera served as a negative control and an unadsorbed anti-J48 whole cell antiserum served as a positive control. Newborn pups were injected subcutaneously within 24 h after birth with

the $LD_{90}$ challenge dose of the respective GBS strain as determined in preliminary $LD_{90}$ experiments. Survival of pups was recorded for the next 72 h (Table 4-2).

## Results

### Determination of $LD_{90}$ for Serotype III δε-positive and δε-negative GBS

Taken together, results of both Takahashi et al. (1998) and Brady et al. (1996) suggested that RDP III-3, δε-positive GBS comprise a more pathogenic lineage of serotype III GBS. Therefore, $LD_{90}$ experiments were conducted to evaluate potential differences in lethality of representative strains in a neonatal mouse model (Rodewald et al. 1992). Four δε-positive and four δε-negative serotype III strains were tested (Figure 4-1). $LD_{90}$ values partitioned into two distinct groups based on the presence or absence of δε antigen. Delta/epsilon-positive strains had $LD_{90}$ values between $4.6 \times 10^2$ and $8 \times 10^3$ cfu, while δε-negative strains demonstrated substantially higher values between $9.8 \times 10^6$ and $6 \times 10^8$ cfu. This large difference in $LD_{90}$ values between the two subsets of serotype III strains supports the hypothesis that δε-positive RDP III-3 organisms are indeed more highly pathogenic.

### Passive Protection Assays

The ability to protect neonatal mouse pups against lethal challenge after passive administration antiserum made against GBS antigens other than capsular polysaccharide to pregnant mothers has been demonstrated (Brodeur et al. 2000, Cheng et al. 2001, 2002, Gravekamp et al. 1999, Larsson et al. 1996, Madoff et al. 1991, 1992, 1994, Stalhammar-Carlemalm et al. 1993). As described in Chapter 2, gel-purified material from J48 (serotype III/δε) demonstrating reactivity with anti-ε and anti-group B antibodies, and corresponding material from DL700 (serotype III), demonstrating reactivity with anti-

group B antibodies were used as immunogens to produce polyclonal rabbit antiserum. The resulting antisera were used in passive protection experiments to determine if antibodies against these immunogens could protect neonatal mice from 90% lethal GBS challenge with the strains from which the immunogens were derived (Table 4-1). A positive control antiserum made against J48 (serotype III/δε) whole cells containing antibodies against the serotype III capsular polysaccharide, a known target of protective antibodies, was able to protect offspring from challenge with either J48 or DL700. Mice that received PBS, preimmune antiserum, or antisera made against J48 or DL700 gel-purified materials were not protected to significant levels. These results suggest that antibodies against ε itself or the corresponding material from a serotype III/δε-negative strain are not able to protect mice from 90% lethal GBS challenge. The reactivity of J48 and DL700 gel-purified material with a group B-specific typing reagent is of interest as antibodies against the group B carbohydrate have been reported to be non-protective. The association of a streptococcal group-specific antigen with a protein component such as the newly identified GBS Srr variants would represent a novel finding. Historically, group-specific carbohydrate antigens were characterized following extraction with hot acid, a technique that would destroy any associated protein components.

**Discussion**

The association of serotype III/δε expression with GBS pathogenesis was investigated further by comparing the $LD_{90}$ values of representative serotype III/δε-positive and δε-negative strains using a neonatal mouse model of infection. The serotype III/δε-positive strains were clearly more pathogenic than serotype III/δε-negative strains in this model with $LD_{90}$ values of the former ranging from approximately

$10^2$ to $10^4$ cfu and of the latter from approximately $10^7$ to $10^9$ (Figure 4.1). In addition to the $LD_{90}$ values themselves, the temporal aspect of this experiment was also interesting. Most of the mice that died after challenge with δε-negative bacteria died within 24 hours post-inoculation, while most mice that died after challenge with δε-positive bacteria died between 24-72 hours. This might suggest that the $LD_{90}$ values for δε-negative strains were so high that the immune system was overwhelmed when that quantity of organisms was encountered. The δε-positive strains demonstrated 3-4 fold lower $LD_{90}$ values and survived in the host for a longer period of time before death of infected animals. It has been shown that serotype III GBS can survive within and disrupt the bactericidal function of macrophages (Cornacchione et al. 1998, Fettucciari et al. 2000, Valentin-Weigand et al. 1996), but δε expression was not evaluated in these studies. It is possible that δε-positive GBS are better able to survive inside host macrophages and therefore persist in the host for a longer period of time.

Several studies suggest that in addition to the capsular polysaccharide, antibodies against other surface structures can also be protective. Madoff et al. (1991) have shown that monoclonal antibodies recognizing the α antigen are protective in mice. Antibodies elicited against the β protein are also protective in mice (Fusco et al. 1997, Madoff et al. 1992, Michel et al. 1991). Antibodies to protein Rib also confer protective immunity (Stalhammar-Carlemalm et al. 1993), as does antiserum recognizing an α-like protein present on serotype V GBS (Lachenauer and Madoff 1996) in a neonatal mouse model. Because antibodies against a number of surface antigens can be protective in mice, the ability of passively administered polyclonal rabbit antisera made against ε-containing material from J48 and corresponding ε-negative material from DL700 to confer

protection in a neonatal mouse model was examined. Both immunogens contained material reactive with a group B streptococcal typing reagent, although only two of the four sugars known to comprise the group antigen were detected in these preparations. Antibodies against the group B carbohydrate have been reported to be non-protective against GBS challenge (Lancefield 1934, 1938). Taken together, results suggest that ε represents additional determinants associated with the group B carbohydrate on serotype III/δε-positive GBS. A possible relationship between the group B carbohydrate and both variants of Srr is also suggested.

While ε is apparently not a target of protective immunity, its utility as a diagnostic marker in conjunction with the δ antigen is strongly suggested by the results of the comparative $LD_{90}$ experiment. The direct role of the δ and ε antigens in the pathogenesis of serotype III GBS remains to be elucidated; however, the expression of these antigens is clearly prognostic of lethality associated with a minimal bacterial load. The association of δ and ε with the highly pathogenic RDP III-3 lineage underscores their clinical relevance and suggests that a rapid test to detect these antigens would be of value in the consideration of treatment options.

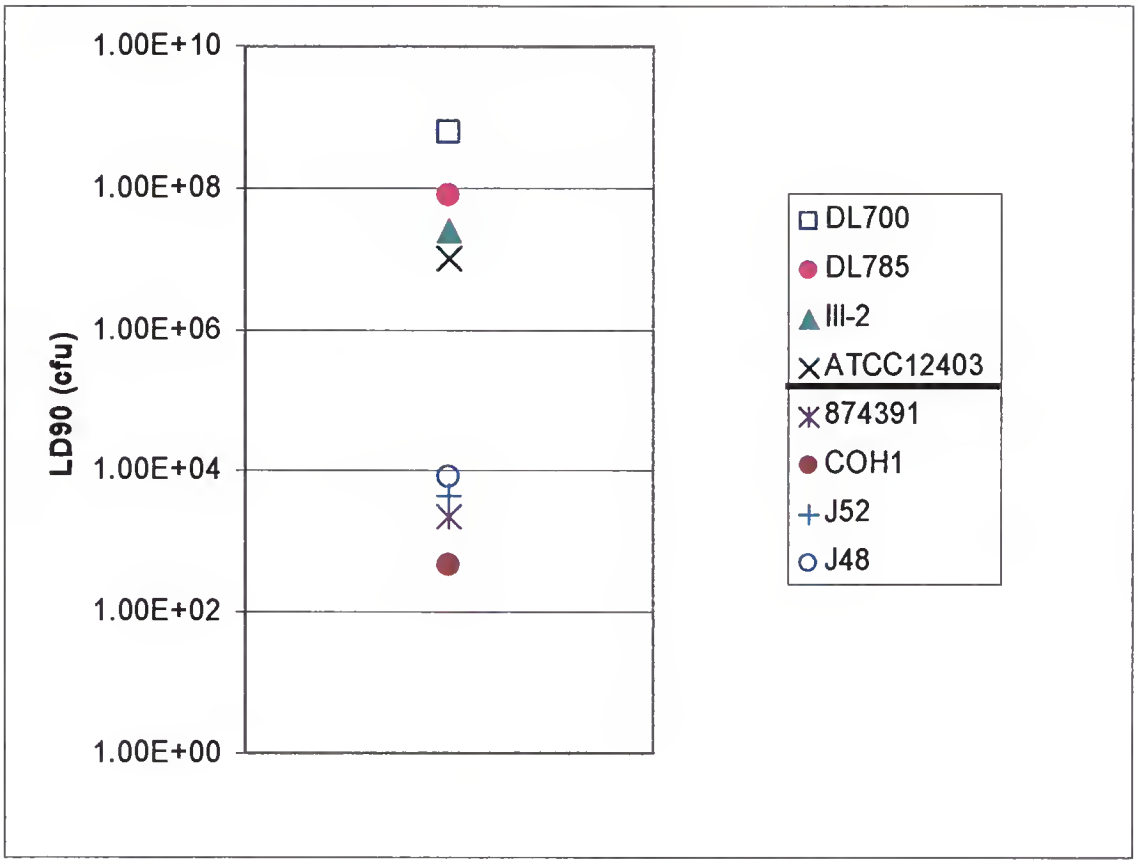



Figure 4-1. Comparison of $LD_{90}$ values of serotype III δε-positive and δε-negative strains. Strains DL700, DL785, 865043, and ATCC12403 are serotype III, δ/ε-negative strains, and 874391, COH1, J52, and J48 are serotype III, δ/ε-positive strains.

Table 4-1. Results of passive protection assays

| Challenge Strain ($LD_{90}$) | Treatment of Dams | Number of neonates surviving GBS challenge (%) |
|---|---|---|
| J48 (III/δε) ($8 \times 10^3$) | PBS | 1/23 (4) |
| | Preimmune rabbit serum | 2/12 (17) |
| | Anti-J48 whole cell antiserum | 25/26 (96) |
| | Anti-J48 gel-purified, ε+ material | 4/17 (24) |
| | Anti- DL700 gel-purified, ε- material | 2/13 (15) |
| DL700 (III) ($6 \times 10^7$) | PBS | 5/24 (20) |
| | Preimmune rabbit serum | 5/22 (22) |
| | Anti-J48 whole cell antiserum | 19/21 (91) |
| | Anti-J48 gel-purified, ε+ material | 2/25 (8) |
| | Anti- DL700 gel-purified, ε- material | 4/27 (15) |

# CHAPTER 5
# CHARACTERIZATION OF GROUP B STREPTOCOCCAL GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE: SURFACE LOCALIZATION, ENZYMATIC ACTIVITY, AND PROTEIN-PROTEIN INTERACTIONS

## Introduction

Cytosolic proteins can be associated with the cell surface of GBS. Glutamine synthetase (Suvarov et al. 1997), Hsp70 (Hamel et al. 1996), α-enolase (Pancholi and Fischetti 1998), glucose-6-phosphate isomerase (Hughes et al. 2002), ornithine carbamoyltransferase (Hughes et al. 2002), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hughes et al. 2002, Pancholi and Fischetti 1992) have all been detected in lysin extracts of GBS. Surface-localized GAPDH has been observed in both prokaryotic and eukaryotic cells. The enzyme exhibits various functions and is able to bind multiple proteins. In prokaryotes, surface-localized GAPDH was originally identified in group A streptococci (GAS) and has subsequently been found in lysin extracts of streptococcal groups B, C, E, G, H, and L (Pancholi and Fischetti 1992). The GAS GAPDH functions as an ADP-ribosylating enzyme (Pancholi and Fischetti 1993) and may be involved in prokaryotic-eukaryotic cell-cell communication (Pancholi and Fischetti 1997). The GAS surface-localized GAPDH also has the capacity to bind a number of human proteins including plasmin(ogen) (D'Costa and Boyle 2000, Winram and Lottenberg 1996), lysozyme, myosin, actin, and fibronectin (Pancholi and Fischetti 1992), which may play a role in the virulence of this organism. The *Staphylococcus aureus* and *Staphylococcus epidermidis* surface-localized GAPDH is a transferrin-binding protein (Moudin and Williams 1999). GAPDH is also expressed on the cell

surface of eukaryotic species *Candida albicans* (Gil-Navarro et al.1997) and *Kluyveromyces marxianus* (Fernandes et al. 1992), and is able to bind both fibronectin and laminin (Gozalbo et al. 1998). Mammalian cells possess a surface-localized GAPDH (Tanner and Gray 1971), (Wooster and Wrigglesworth 1976), where it is involved in membrane fusion (Glaser and Gross 1995).

In the course of characterizing the δ and ε surface antigens associated with serotype III GBS, a protein in a bacterial sonic extract homologous to other prokaryotic surface-localized GAPDH proteins was identified. There have been preliminary reports of GAPDH surface expression by GBS (Hughes et al. 2002, Pancholi and Fischetti 1992). A GBS gene encoding a GAPDH homolog (*gapC*) was identified by several investigators (Fontaine et al. 2002, Glaser et al. 2002, Tettelin et al. 2002). However, little functional information was reported. The GAPDH molecule which was detected in a sonic extract of serotype III/δε strain J48 has high nucleotide and amino acid identity (87%) to the plasmin-binding protein (Plr) of GAS strain 64/14 (Lottenberg et al. 1992), as well as a number of other bacterial GAPDHs (Gase et al. 1996, Nelson et al. 2001), including those identified in GBS (Fontaine et al. 2002, Glaser et al. 2002, Tettelin et al. 2002). To begin to characterize the GBS GAPDH, representative strains of five different serotypes were assayed for GAPDH enzymatic activity. DNA hybridization using a *gapC* probe was used to determine if a single or multiple genes within the GBS genome encode both the cytosolic and surface-localized forms of the protein. Western blot analysis was utilized to determine if GBS GAPDH is able to bind lys-plasminogen, glu-plasminogen, and fibrinogen. An ELISA assay was used to determine if fluid-phase GAPDH could bind to immobilized lys-plasminogen, glu-plasminogen, actin, and fibrinogen. The results of

these experiments offer partial characterization of the GBS surface-localized GAPDH. The results for a majority of these experiments have been published previously (Seifert et al. 2003).

## Materials and Methods

### Bacterial Strains and Growth Conditions

Group B streptococcal (GBS) strains used in this study were A909 (serotype Ic/αβγδ), ss617 (serotype Ia), ss618 (serotype Ib/αβγ), 9B200 (serotype II/αγ), J48 (serotype III/δε), J52 (serotype III/δε), PEH (serotype III/δε), U1378 (serotype III), COH1 (serotype III/δε), COH1-13 (serotype III/δε/ acapsular mutant), DL686 (serotype III), and DL700 (III). Strains A909, ss617, and ss618 were obtained from Richard Facklam at the Centers for Disease Control and Prevention (Atlanta, GA, USA). Strains 9B200, J48, J52, PEH, and U1378 were clinical isolates from Shands Hospital, University of Florida. Strains COH1 and COH1-13 were generous gifts from Dr. Craig Rubens (Children's Hospital Regional Medical Center, Seattle, WA, USA). COH1-13 is an acapsular mutant of the parent strain COH1 (Rubens et al. 1987, Wessels et al. 1989). Strains DL686 and DL700 were clinical isolates kindly provided by Dr. Daniel Lim (University of South Florida, Tampa, FL, USA. Group A streptococcal strain 64/14 (Lottenberg et al. 1992) was used as a positive control in the GAPDH enzymatic activity assays. All streptococci were grown to late exponential phase in Todd-Hewitt Broth (Becton Dickinson, Cockeysville MD) at 37°C without shaking.

### Immunological Reagents, Cytoskeletal and Extracellular Matrix Proteins

IgG monoclonal antibodies specific for the recombinant Plr protein (GAPDH) of GAS strain 64/14 were gifts from Dr. Michael Boyle, Medical College of Ohio, Toledo,

Ohio (D'Costa et al. 1997). Human plasmin, human glu-plasminogen, human lys-plasminogen, and IgG monoclonal antibodies specific for these proteins were obtained from American Diagnostica, Inc. (Greenwich, CT). Human fibronectin was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Human fibrinogen, myosin (calcium activated from rabbit muscle), actin (rabbit muscle), glyceraldehyde-3-phosphate, and $NAD^+$ were obtained from Sigma (St. Louis, MO). Monoclonal antibodies specific for fibrinogen (IgG), myosin (IgG), actin (IgG), and fibronectin (IgM) were also obtained from Sigma (St. Louis, MO). Affinity-purified horseradish peroxidase (HRP)-labeled goat-anti-mouse (H+L chain) was obtained from ICN Biomedicals, Inc (Aurora, OH).

**Identification of GAPDH in Sonic Extracts of GBS Following Anion-Exchange Chromatography**

Sonic extracts of GBS strain J48 (serotype III/δε) were prepared by sonicating bacteria three times for 30 s in 20 mM Tris-HCl pH 8.0 with P-800 Potter's glass beads (1:1:1, wet weight per volume per weight) on ice with a Sonic 300 Dismembrator (ARTEK Systems Corporation, Farmingdale, NY). The glass beads and bacterial debris were removed from the sonicated mixture by centrifugation at 10 X g for 5 min. Wall membranes were removed in a Beckman L7-55 Ultracentrifuge at >100,000 × *g* for 2 h. The non-pelleted material was collected and filtered through 0.2 mm Acrodiscs (Gelman Sciences). The soluble bacterial sonic extract was diluted 1:3 in 20 mM Tris-HCl pH 8.0 and loaded onto an UNO-Q1 (Bio-Rad) anion-exchange column. Unbound material was removed from the column by washing with 2.5 column volumes of 20 mM Tris-HCl pH 8.0. Bound molecules were eluted from the column using a stepwise gradient of 0-1 M NaCl of 0.05 M NaCl increments in 20 mM Tris-HCl pH 8.0. Anion-exchange column

fractions eluted from the column with 0.2 M NaCl contained δ and ε antigenic reactivities and were chosen for further study. These fractions were subsequently demonstrated to also contain serotype III polysaccharide and material reactive with a group B streptococcal typing reagent.

Anion-exchange column fractions were separated on a 7.5% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane (Protran®, Scleicher & Schuell), and stained for protein with AuroDye Forte™ (Amersham). In an attempt to identify and characterize high molecular weight proteins, a protein band of ~173.5 kDa was excised from the membrane and subjected to N-terminal sequencing by the ICBR using automated Edman chemistry. The unambiguous sequence obtained was VVKVGINGFGRIGRLAFRRIQN. BLAST analysis (Altschul et al. 1997) demonstrated 100% identity with the amino terminus of the plasmin-binding protein Plr from GAS strain 64/14 (Lottenberg et al. 1992), a molecule known to represent surface-localized GAPDH.

**Western Immunoblot Detection of GBS GAPDH**

Anion-exchange column fractions known to contain the sequenced ~173.5 kDa band were separated on 7.5% SDS-polyacrylamide gels, transferred onto a nitrocellulose membrane (Protran®, Scleicher & Schuell), and blocked with PBS containing 0.3% Tween-20 (PBS-Tw). The membrane was incubated overnight at room temperature with a monoclonal antibody raised against GAPDH of *S. pyogenes* (anti-Plr). After washing, the blot was incubated with affinity-purified HRP-labeled goat-anti-mouse (H+L chain) at a 1:1000 dilution (ICN Biomedicals, Inc, Aurora, OH) for 2 hours at room temperature, washed, and developed with 8 mL of 4-chloro-1-naphthol solution (7 mL PBS, 1 mL 4-

chloro-1-naphthol [Sigma; 3 mg/mL in methanol], 8 mL of 30% hydrogen peroxide [Fisher Scientific]).

**Detection of GAPDH on GBS Whole Cells by ELISA**

GBS strains A909 (serotype Ic/αβγδ), ss617 (serotype Ia,) ss618 (serotype Ib), J48 (serotype III/δε), and DL700 (serotype III) were assayed for the presence of surface-localized GAPDH by ELISA. Costar High Biding plates (Costar) were used. Sample wells were coated overnight at 4°C in a moist chamber with 100 mL of 0.1M carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide and approximately $10^7$ streptococcal whole cells. The plates were blocked with PBS containing 0.03% Tween-20 (PBS-Tw). Test wells were incubated with 100 mL of monoclonal anti-Plr antibody (1:1000 dilution in PBS) for 2 hat 37°C. After washing 3 X with 300 mL PBS-Tw, 100 mL of affinity-purified HRP-labeled goat-anti-mouse (H+L) secondary antibody (1:1000 dilution in PBS) (ICN Biomedicals) was added to the wells for 2 hours at 37°C. Plates were washed, and developed with o-phenylenediamine (OPD) in 0.1M citric acid/0.2M sodium phosphate buffer in the presence of 0.03% hydrogen peroxide. $OD_{450}$ of test wells was determined using a Model 550 microplate reader (Bio-Rad). Control wells included incubation of streptococcal whole cells with secondary antibody alone to determine background $OD_{450}$ values.

**PCR Amplification and Cloning of the Gene Encoding GAPDH from GBS Strain J48**

Chromosomal DNA from J48 (serotype III/δε) was isolated as described by Nagano et al. (1991) and further purified using CsCl gradients. DNA encoding GAPDH (*gapC*) was amplified by PCR from J48 chromosomal DNA using the forward primer 5' ATGGTAGTTAAAGTTGGTATTA 3' and the reverse primer 5'

TTATTTAGCAATTTTTGCGAAGTA 3' as determined from the *plr* sequence of GAS strain 64/14 (Lottenberg et al. 1992). DNA was first denatured at 94°C for 1 min. Thirty amplification cycles were performed as follows: 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of extension at 72°C, followed by one cycle of 5 min at 72°C. The PCR product was analyzed by gel electrophoresis in 0.7% agarose. The amplified product was ligated to the TOPO-TA cloning vector, pCR2.1 (Invitrogen, Carlsbad, CA) and used to transform *E. coli* strain INVαF' according to the manufacturer's protocol. Transformants were selected for on Luria-Bertani- (LB) agar plates containing kanamycin (50 μg/mL) and X-gal (75 μg/mL). White colonies were selected for further analysis. Transformant KS100 was selected for further study and harbored a 4.9 kb plasmid. The plasmid was purified using the Qiagen Plasmid Maxi Kit according to the manufacturer's protocol and found to contain a 1.0 kb insert.

The GBS DNA insert in pKS100 was sequenced by the ICBR using the universal primers supplied with the TOPO-TA Cloning Kit (Invitrogen). The sequence obtained was analyzed using BLAST analysis (Altschul et al. 1997). The GenBank accession number of the primary nucleotide sequence of the GBS strain J48 *gapC* is AF338416. The nucleotide sequence was 87% identical to the *plr* (plasmin-binding protein) sequence of *S. pyogenes* strain 64/14 (GenBank accession number M95569). The nucleotide sequence was 99% identical to *gapC* from GBS strain ATCC 27541(GenBank accession number AF421900) (Fontaine et al. 2002) and *gapC* in the published genome sequences of NEM316 (Glaser et al. 2002) and 2603 V/R (Tettelin et al. 2002), listed as *gbs1811* and *SAG1768*, respectively.

**Southern Hybridization to Evaluate *gapC* Copy Number in GBS**

DNA hybridization was performed by the method of Southern (Sambrook et al. 1989). Chromosomal DNA from all GBS strains tested was isolated as described by Nagano et al. (Nagano et al. 1991) and further purified on CsCl gradients. Two micrograms of genomic DNA from each of the six GBS strains tested was digested with *Ssp*I (Promega, Madison, WI) at 37°C overnight. The digested DNA was separated by electrophoresis through a 0.7% agarose gel and transferred to a nylon membrane (Boehringer Mannheim) by a capillary blot procedure according to the manufacturer's instructions. The membrane was probed with J48 (serotype III/δε) *gapC* labeled during PCR amplification with Digoxigenin-11-dUTP (DIG-dUTP, Boehringer Mannheim) at 37°C overnight. The blot was developed according to the manufacturer's instructions.

**GAPDH Activity Associated with GBS Whole Cells**

The assay for GAPDH activity was carried out according to the method of Ferdinand (Ferdinand 1964) with minor modifications. Suspensions of $10^8$ cfu/100 µl of each GBS strain and the positive control GAS strain 64/14 were incubated with 7 µl glyceraldehyde-3-phosphate (G-3-P) (50 mg/mL; Sigma), 100 µl $NAD^+$ (10 mM; Sigma), and assay buffer consisting of 40 mM triethanolamine, 50 mM $Na_2HPO_4$, and 5 mM EDTA (pH 8.6) in a final volume of 1.0 mL. After incubation for 30 min at 37°C, bacteria were removed by centrifugation, and the supernatants were tested for the relative levels of NADH by measuring the absorbance at 340 nm. As controls, suspensions in which either streptococcal cells or G-3-P was omitted were used. All assays were performed in triplicate.

**Protein Binding Characteristics of GAPDH**

Anion-exchange column fractions containing GAPDH were electrophoresed through 10% SDS-polyacrylamide preparatory gels and electroblotted onto a nitrocellulose membrane (Protran®, Scleicher & Schuell). The membrane was cut into strips, and individual strips were stained for protein with colloidal gold solution (Membragold, Diversified Biotech, Boston MA) or incubated with 50 micrograms of gly-plasminogen, lys-plasminogen, plasmin, myosin, actin, or fibrinogen in 1 mL of PBS overnight at room temperature. Each membrane strip was then incubated with monoclonal antibodies specific for the protein being tested for 2 h at room temperature, washed, and incubated with affinity-purified HRP-labeled goat-anti-mouse (H+L) (1:4000 dilution) (ICN Biomedicals, Inc) for 2 h at room temperature. Optimal dilutions of each monoclonal antibody had been previously determined in preliminary Western blot experiments utilizing each protein of interest. The membrane strips then were developed in 4-chloro-1-naphthol (Sigma) (3 mg/mL in methanol). Controls for this experiment included a membrane strip reacted with monoclonal anti-Plr antibody to identify the GAPDH bands, a membrane strip incubated with HRP-labeled goat-anti-mouse (H+L) secondary antibody only, and membrane strips reacted with each monoclonal antibody alone to ensure against non-specific binding to proteins contained in the column fractions.

Extracellular matrix and cytoskeletal proteins lys-plasminogen, glu-plasminogen, plasmin, myosin, actin, fibrinogen, and fibronectin were coated on 96-well ELISA plates at 100 ng/well in coating buffer (100 µl of 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide). Plates were blocked with PBS-Tw. Test wells were incubated with 100 µl of a column fraction known to contain GAPDH diluted 1:100 in 20

mM Tris-HCl pH 8.0. The ELISA plate was washed 3 times with PBS-Tw to remove unbound proteins and bound GAPDH was detected by incubation with anti-Plr monoclonal antibody (1:5000), HRP-labeled goat-anti-mouse conjugate (1:5000), and development with o-phenylenediamine (OPD) in 0.1M citric acid/0.2M sodium phosphate buffer in the presence of 0.03% hydrogen peroxide. $OD_{450}$ was determined using a Model 550 microplate reader (Bio-Rad). Assays were performed in triplicate and values are reported as the average of the three assays ± SEM.

## Results

### Identification of Surface-Localized GBS GAPDH

An anion-exchange column fraction of a sonic extract of GBS strain J48 (serotype III/δε) that contained anti-group B reactive carbohydrate, capsular polysaccharide, the δ and ε antigens, and other proteins was separated on a 10% SDS-polyacrylamide gel, transferred onto nitrocellulose, and stained for protein (Figure 5-1, Lane A). A protein band migrating at ~173.5-kDa was excised and subjected to N-terminal sequencing. The unambiguous sequence VVKVGINGFGRIGRLAFRRIQN was obtained. When compared to known sequences in the translated GenBank database, identity (100% of 22 amino acids) was observed with the plasmin-binding protein Plr from GAS strain 64/14 (Lottenberg et al. 1992), a molecule known as surface-localized glyceraldehyde-3-phosphate-dehydrogenase. A monoclonal antibody to recombinant Plr protein of GAS strain 64/14 was used to probe a Western immunoblot of the column fraction (Figure 5-1, Lane B). The anti-Plr monoclonal antibody bound to the ~173.5 kDa GBS protein band identified by N-terminal sequencing as GAPDH. Anti-Plr also reacted with a doublet of approximately 90 kDa as well as a ~41 kDa band that is equivalent to the approximate

molecular mass of a single GAS GAPDH subunit (Pancholi and Fischetti 1992, Winram and Lottenberg 1996). The ~173.5 kDa band chosen for N-terminal sequencing most likely represents the tetrameric form of the molecule. The anti-Plr monoclonal antibody was also in an ELISA experiment to determine if GAPDH is present on the surface of GBS with GAS strain 64/14 used as the positive control (Figure 5-2). The anti-Plr monoclonal antibody bound to all strains tested, including representatives of serotypes Ia, Ib, Ic, and III. Other serotypes were not tested. These results suggest that GAPDH exists as a surface-localized molecule on GBS.

**Cloning of GBS *gapC* and Sequence Analysis**

Using the GAS *plr* sequence as a guide, primers were designed to amplify the *gapC* ORF from GBS strain J48 (serotype III/δε) chromosomal DNA by PCR. The amplified DNA was ligated to the vector pCR2.1 and was subsequently purified and sequenced. The amplified product contained a 1008-bp ORF. BLAST analysis (Altschul et al. 1997) indicated were 87% identical to GAS *plr,* both in terms of nucleotide and predicted amino acid sequence. The nucleotide sequence was 99% identical to *gapC* from GBS ATCC 27541 (Fontaine et al. 2002) and to *gapC* in the published genome sequences of NEM316 (Glaser et al. 2002) and 2603 V/R (Tettelin et al. 2002), listed as *gbs1811* and *SAG1768* respectively.

**GAPDH Activity is Associated with the Group B Streptococcal Cell Surface**

The high level of homology of *gapC* with *plr* of GAS strain 64/14 suggested that GBS whole cells should catalyze the conversion of glyceraldehyde-3-phosphate (G-3-P) to 1, 3-diphosphoglycerate in the presence of inorganic phosphate and, in the process, generate NADH from $NAD^+$. GBS strains representative of serotypes Ia, Ib, Ic, II, and III were assayed for their GAPDH activity by monitoring the formation of NADH at 340

nm. GAS strain 64/14 was used as the positive control for this experiment. The data shown in Figure 5-3 demonstrate that intact GBS cells possess GAPDH activity. Enzymatic activity varied between, and within serotypes, with strain A909 having the lowest activity and strain COH1 having the highest activity.

**The Genome of Group B Streptococci Contains a Single Copy of *gapC***

Organisms that express GAPDH may possess more than one *gapC* gene (Alefounder and Perham 1989, Holland et al. 1983). Therefore, DNA hybridization analysis by the method of Southern (Sambrook et al. 1989) was performed on chromosomal DNA digested with *Ssp*I from six different strains of GBS using a probe consisting of the 1-kb PCR amplified *gapC* ORF to test whether the GBS genome contains a single or multiple copies of the *gapC* gene (Figure 5-4). In all instances, the probe hybridized with a single fragment (Lanes 1-6). These results are consistent with a single copy of the *gapC* gene in all strains tested. This is analogous to the situation for GAS *plr* (Winram and Lottenberg 1996) and consistent with the presence of a single copy of *gapC* in the published genomic sequences of a serotype III and serotype V GBS strain (Glaser et al. 2002, Tettelin et al. 2002).

**Binding of Extracellular Matrix and Cytoskeletal Proteins by Immobilized GAPDH**

Surface-localized GAPDH has been shown to bind a number of eukaryotic proteins (D'Costa and Boyle 2000, Moudin and Williams 1999, Pancholi and Fischetti 1992, Winram and Lottenberg 1996). The ability of lys-plasminogen, glu-plasminogen, plasmin, myosin, actin, fibrinogen, and fibronectin to interact with GAPDH immobilized on nitrocellulose was tested by a Western immunoblot technique in which binding was assessed by incubating the membrane with the protein of interest, followed by a monoclonal antibody against the test protein, followed by an HRP-labeled secondary

antibody (Figure 5-5). Lys- and glu-plasminogen bound to both the tetrameric and monomeric forms of GAPDH, while plasmin, myosin, actin, and fibrinogen did not react with either form. Bands corresponding to GAPDH did not react with any of the monoclonal antibodies in the absence of test protein or with secondary antibody only (data not shown).

### Reactivity of GAPDH with Immobilized Extracellular Matrix and Cytoskeletal Proteins

An ELISA assay was used to determine if the interactions of GBS GAPDH with extracellular matrix and cytoskeletal proteins were different depending on which moiety was immobilized. Proteins of interest were coated onto ELISA plate wells, and reactivity with GAPDH was evaluated following incubation with a GAPDH-containing column fraction. Bound GAPDH was detected using the anti-Plr monoclonal antibody. In this assay, fluid-phase GAPDH was able to interact with actin and fibrinogen, in addition to lys- and glu-plasminogen (Figure 5-6). Fluid-phase GAPDH did not interact with immobilized plasmin, myosin, or fibronectin.

## Discussion

GAPDH was identified as a surface-expressed molecule of GBS during analysis of high molecular weight antigens in anion-exchange column fractions that also contained group-specific antigen, capsular polysaccharide, and the δ and ε antigens (Figure 5-1). GBS GAPDH was first identified by N-terminal sequencing as a ~173.5 kDa protein, presumably the tetrameric form on an SDS-polyacrylamide gel run under non-reducing conditions. A ~41-kDa protein band, presumably the monomeric form, was subsequently identified by Western immunoblot using an anti-Plr monoclonal antibody made against GAPDH from *S. pyogenes*. In GAS, the enzymatically active form of GAPDH is a

tetramer comprised of ~41-kDa subunits. The GAS *plr* and GBS *gapC* share 87% homology and are both predicted to encode proteins of ~36 kDa. The apparent molecular mass of GAS native and recombinant GAPDH monomer by SDS-PAGE has been reported to be ~41 kDa (Pancholi and Fischetti 1992, Winram and Lottenberg 1996). This is similar to what was observed for the GBS GAPDH. It is unclear why anti-Plr reactivity was also detected with a 90-kDa doublet. This may represent a previously unrecognized intermediate form of GAPDH. The tetrameric form of GAS GAPDH has been reported to migrate at 150 kDa (Pancholi and Fischetti 1992) This is smaller than the ~173.5 kDa observed on Western blots for GBS GAPDH. It is possible that extraction of surface molecules by sonication does not completely disrupt interactions of tetrameric GBS GAPDH with other surface structures, such as cell wall components and polysaccharide capsule. It is also possible that the surface-localized form of the GBS active enzyme is glycosylated, as is the case with *Kluyveromyces marxianus* (Fernandes et al. 1992). Analysis for potential post-translational modifications of GBS GAPDH has not yet been performed, but could explain its observed migration at ~173.5 kDa. Reactivity of the anti-Plr monoclonal antibody with GBS whole cells suggests that in GBS GAPDH can exist as a surface-localized protein (Figure 5-2). GAPDH has been observed in lysin extracts of a number of streptococcal species (Hughes et al. 2002, Pancholi and Fischetti 1992), including GBS.

Comparison of the GBS *gapC* and GAS *plr* sequences revealed 87% identity (Winram and Lottenberg 1996). Predicted amino acid sequences were also 87% identical. Similar to GAS *plr*, the predicted protein encoded by GBS *gapC* lacks a signal sequence and has no apparent cell wall-spanning or membrane-anchoring motifs (Lottenberg et al.

1992, 1994). This is in contrast to many other known gram-positive cell wall proteins (Navarre and Schneewind 1999). It is unknown at this time how streptococcal GAPDH is anchored to the cell surface or how it is translocated through the cell membrane.

The enzymatic activity of GAPDH depends on its tetrameric conformation (Walker et al. 1980). GBS whole cells demonstrated the presence of enzymatically active GAPDH (Figure 5-3). All eleven GBS strains tested demonstrated enzymatic activity, although the activity varied between and within the five serotypes evaluated. An acapsular mutant (COH1-13) had much lower GAPDH enzymatic activity than the encapsulated parent, COH1, suggesting that the degree of encapsulation of GBS may play a role in the amount of surface-localized GAPDH and (or) associated enzymatic activity. The amount of anti-Plr monoclonal antibody bound to GBS whole cells was comparable between strains that had large differences in observed enzymatic activity. The reason(s) for this apparent discrepancy is (are) not known. The anti-Plr antibody likely recognizes an epitope unrelated to the active site; therefore, its binding would not necessarily predict functional activity. It is also possible that there may be differences in the number of enzymatically active tetrameric GAPDH molecules on the cell surface, which may depend on the degree of encapsulation of GBS. It is possible that the polysaccharide capsule may lend stability to the GBS tetrameric molecule.

Prokaryotic and eukaryotic organisms may possess single or multiple genes encoding GAPDH (Alefounder and Perham 1989, Holland et al. 1983). Southern hybridization results (Figure 5-4) indicate that GBS possess a single gene that encodes both cytosolic and surface-localized GAPDH. This is the consistent with the published genome sequences of a serotype III and a serotype V GBS strain (Glaser et al. 2002,

Tettelin et al. 2002), and with *plr* encoding GAPDH of GAS (Winram and Lottenberg 1996). The possibility remains, however, that a second, highly divergent *gap* gene may be present on the GBS chromosome that was not detectable by DNA hybridization using the *gapC* probe. This would parallel the situation with *Saccharomyces cerevisiae*, which has three GAPDH genes (Holland et al. 1983), and *E. coli*, which has two GAPDH genes (Alefounder and Perham 1989).

Group A streptococci are able to bind a number of eukaryotic proteins including plasmin (Winram and Lottenberg 1996), myosin, actin (Pancholi and Fischetti 1992), lys-plasminogen, glu-plasminogen, and fibronectin depending on the strain tested (D'Costa and Boyle 2000, Pancholi and Fischetti 1992). Therefore, the ability of GBS GAPDH to interact with a number of these proteins was tested (Figures 5-5 and 5-6). Both lys- and glu-plasminogen was able to bind to immobilized GBS GAPDH, while plasmin, myosin, actin, and fibrinogen were not. Since only strain J48 (serotype III/δε) was used for the binding studies it is possible that other GBS strains would demonstrate different protein binding properties. In addition to GAPDH, plasminogen has been shown to interact with a number of streptococcal proteins including α-enolase (Pancholi and Fischetti 1998), plasminogen-binding group A streptococcal M-like protein (PAM) (Wistedt et al. 1998), plasminogen activator uberis (PauA) (Rosey et al. 1999), streptokinase (Kim et al. 2000), and M-proteins (Svensson et al. 1999). Therefore, it was not surprising to observe interaction of lys- and glu-plasminogen with GBS proteins other than GAPDH. When extracellular matrix and cytoskeletal proteins themselves were immobilized on ELISA plates, GAPDH was shown to bind to actin and fibrinogen in addition to lys- and glu-plasminogen. The results indicate that certain protein-protein interactions depend on

which binding partner is immobilized. Although the group A *plr* and group B *gapC* demonstrate high levels of homology, the surface-localized GAPDH molecules from these two species bind to different extracellular matrix and cytoskeletal proteins. Binding of host macromolecules to GAPDH on the cell surface has been suggested to contribute to streptococcal virulence. For example, Boyle et al. (1997) have shown that when plasmin is bound to surface-localized Plr of GAS the enzyme is no longer regulated by its physiological inhibitor $\alpha_2$-antiplasmin, a property consistent with enhanced invasion. Streptococcal GAPDH also appears to be a target of protective immunity. Immunization with recombinant GapC from *Streptococcus uberis* but not *Streptococcus dysgalactiae* was protective against bovine mastitis after heterologous challenge (Fontaine et al. 2002).

Results presented in this chapter demonstrated that a single copy of *gapC* is present in multiple strains of GBS, that GBS GAPDH enzymatic activity is associated with whole bacterial cells suggesting surface localization, that the molecule is enzymatically active on representatives of serotypes Ia, Ib, Ic, II, and III GBS, and that GBS GAPDH can interact with extracellular matrix and host proteins including lys-plasminogen, glu-plasminogen, actin, and fibrinogen. Additional experiments are required to determine if any of the observed protein-binding interactions contribute to adhesion and (or) invasion during GBS infections and if GAPDH is a factor in GBS pathogenesis.

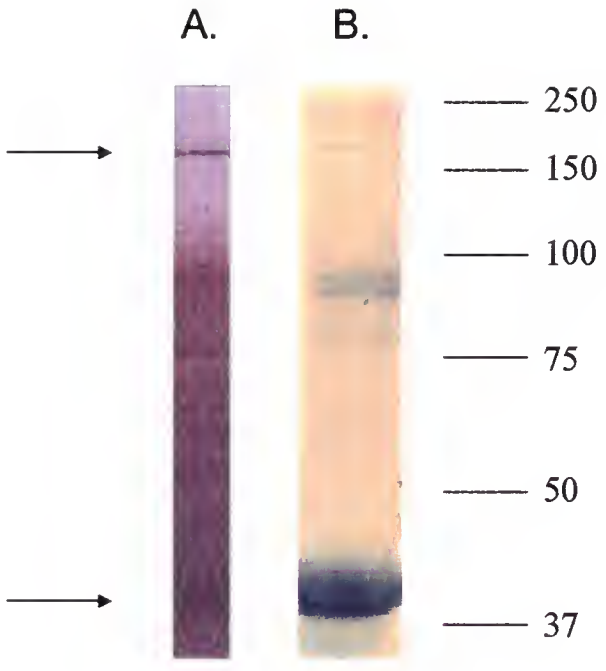



Figure 5-1. Protein stain and Western immunoblot analysis of GBS GAPDH. An anion-exchange column fraction containing surface antigens from GBS strain J48 was separated on duplicate 7.5% SDS-polyacrylamide gels and electroblotted onto a polyvinyldene or nitrocellulose membrane. A: AuroDye Forte™ (protein stain). The 173.5 kDa band (top arrow) was subjected to N-terminal sequencing. B: Western blot incubated with anti-Plr monoclonal antibody. Presumptive monomeric and tetrameric forms of glyceraldehyde-3-phosphate dehydrogenase are indicated by arrows.

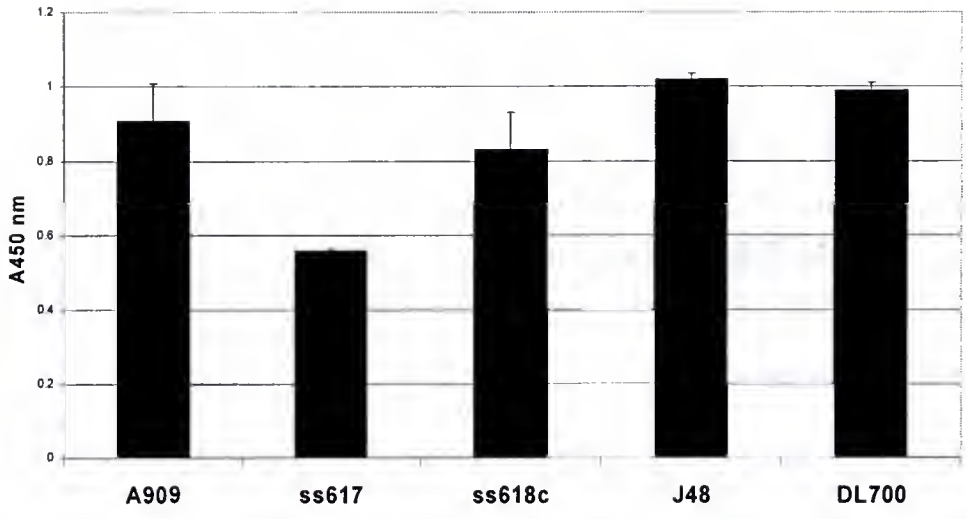



Figure 5-2. Detection of surface-localized GBS GAPDH by ELISA using an anti-Plr monoclonal antibody. Assays were done in triplicate and error bars represent standard error of the mean. Streptococci tested included the positive control group A streptococcus strain 64/14 and GBS strains A909 (serotype Ic/αβγδ), ss617 (serotype Ia), ss618 (serotype Ib/αβγ), J48 (serotype III/δε), and DL700 (serotype III).

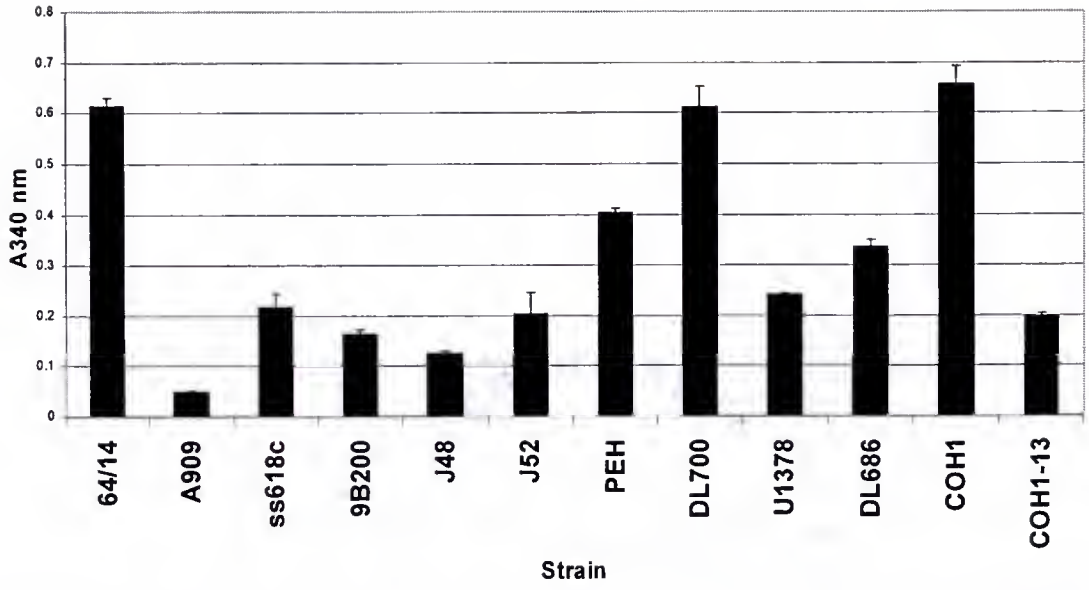



Figure 5-3. Evaluation of GAPDH enzymatic activity associated with GBS whole cells. Assays were performed in triplicate, and error bars represent the SEM. Group A streptococcus strain 64/14 was used as the positive control. Group B streptococcal strains tested were A909 (serotype Ic/αβγδ), ss618 (serotype Ib), 9B200 (serotype II/αγ), J48 (serotype III/δε), J52 (serotype III/δε), PEH (serotype III/δε), DL700 (serotype III), U1378 (serotype III), DL686 (serotype III), COH1 (serotype III/δε), and COH1-13 (serotype III/δε/acapsular mutant of COH1).

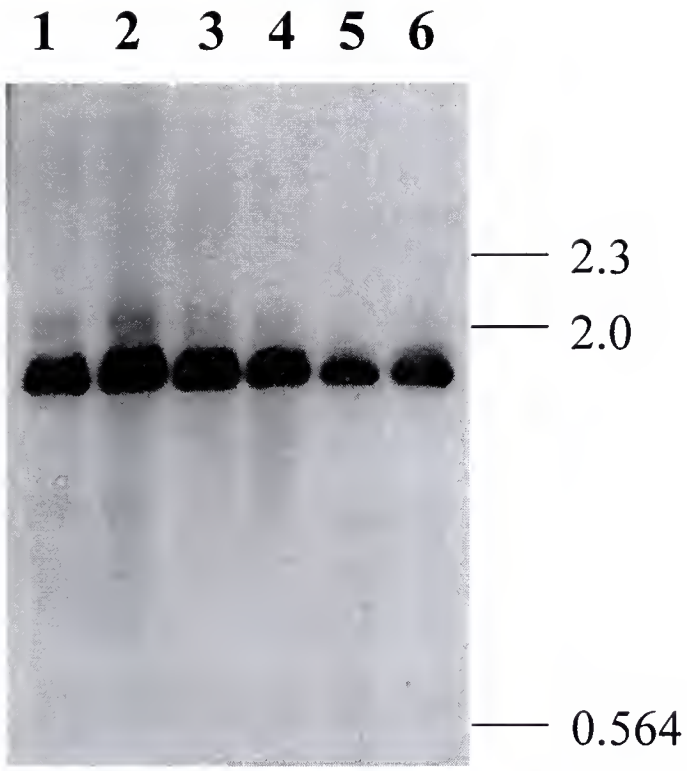



Figure 5-4. Evaluation of GBS *gapC* copy number by Southern hybridization. Chromosomal DNA from 6 strains of GBS was digested with *Ssp*I and incubated with a DIG-labeled probe consisting of the *gapC* amplified from strain J48. Lanes: 1, A909 (serotype Ic/αβγδ), 2, ss618 (serotype Ib/αβγ), 3, 9B200 (serotype II/αγ), 4, J48 (serotype III/δε), 5, J52 (serotype III/δε), 6, PEH (serotype III/δε).

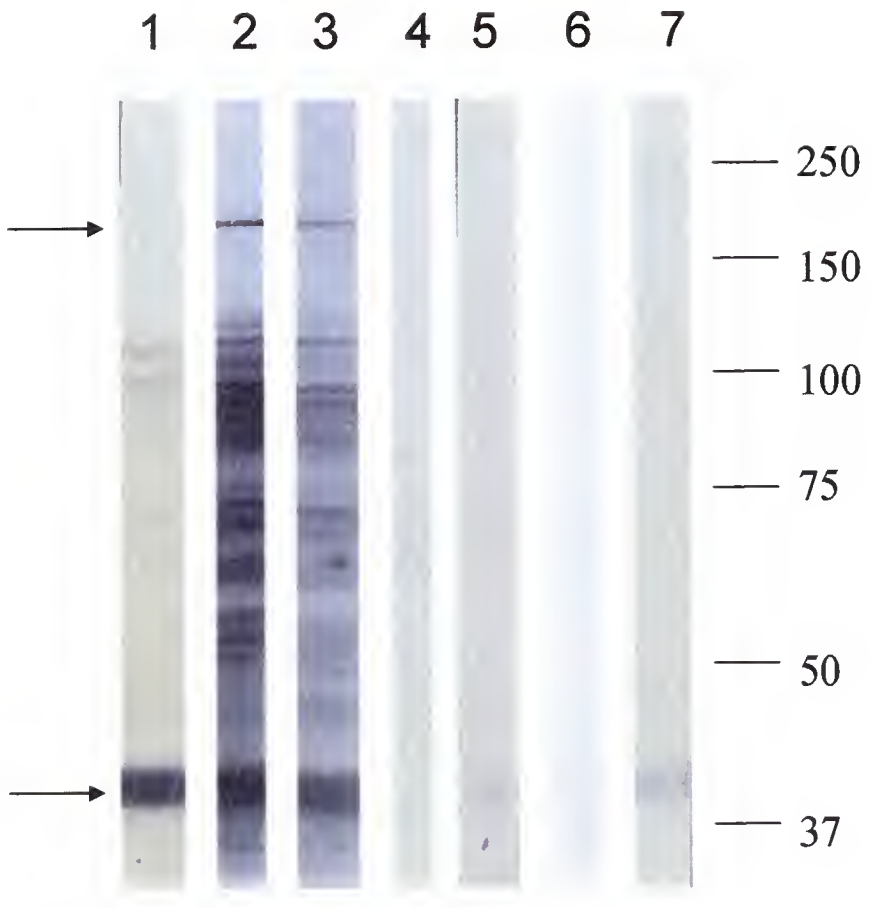



Figure 5-5. Western immunoblot analysis to detect binding of extracellular matrix and cytoskeletal proteins to immobilized GBS GAPDH. A column fraction containing GBS GAPDH was electrophoresed on a 7.5% SDS-polyacrylamide preparatory gel. The proteins were electroblotted onto a nitrocellulose membrane and the membrane was cut into strips. Lane 1 shows reactivity with anti-Plr monoclonal antibody. Arrows indicate the putative monomeric (41 kDa) and tetrameric (173.5 kDa) forms of GAPDH. Individual strips were also incubated with lys-plasminogen (lane 2), glu-plasminogen (lane 3), plasmin (lane 4), myosin (lane 5), actin (lane 6), or fibrinogen (lane 7), followed by monoclonal antibodies to the individual proteins.

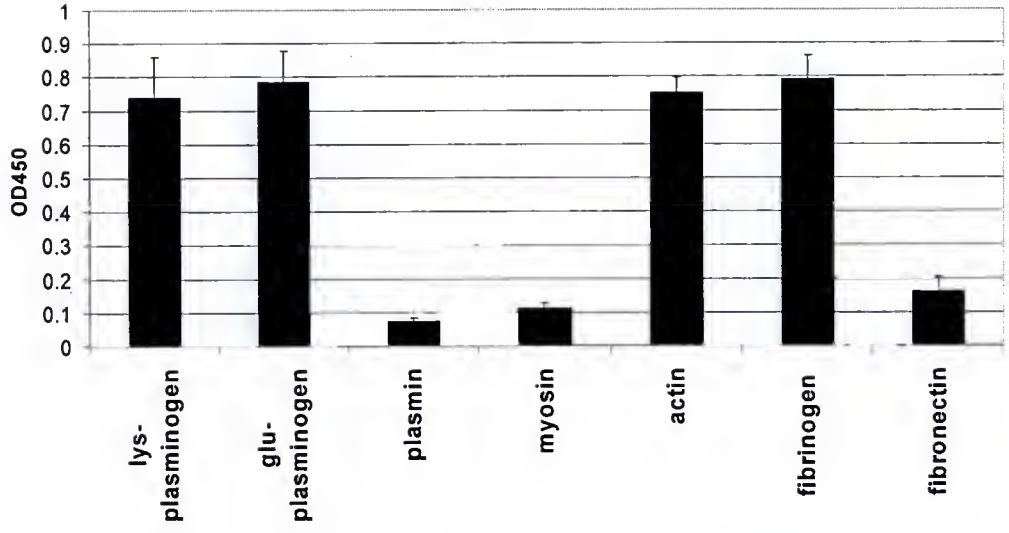



Figure 5-6. ELISA to detect binding of GBS GAPDH to immobilized extracellular matrix and cytoskeletal proteins. Assays were done in triplicate and error bars represent the standard error of the mean.

# CHAPTER 6
# SUMMARY AND CONCLUSIONS

Group B streptococci (GBS) are a major cause of neonatal sepsis (Baker and Edwards 1994) and are important pathogens in both immunocompromised adults and in adults with underlying health conditions such as diabetes, cancer, or cirrhosis of the liver (Farley 2001). To date, GBS have been subdivided into nine serotypes based on the linkages of four sugars that comprise the capsule. The capsular polysaccharide has been shown to be an important virulence factor of GBS (Marques et al. 1992, Rubens et al. 1987, Wessels et al. 1989). Serotype III GBS have been isolated from cases of early-onset disease and are responsible for approximately 90% of all late-onset infections (Baker and Edwards 1994), with meningitis being a common clinical manifestation that results in a substantial number of survivors suffering permanent neurological damage.

In addition to classifying GBS according to polysaccharide capsule, serotype III GBS isolates can be further classified based on restriction digest patterns (RDPs) of chromosomal DNA into four distinct lineages, designated RDP types III-1, 2, 3, and 4 (Takahashi et al. 1998). The majority (91%) of serotype III invasive neonatal isolates have been reported to be RDP III-3, suggesting that these strains may represent a more virulent lineage of serotype III GBS; however, the phenotype of RDP III-3 strains compared to the other RDP types has not been elucidated.

By adsorbing polyclonal rabbit antiserum made against GBS whole cells, two novel antigens, δ and ε, were discovered (Brady et al. 1988). These antigens were found to be associated with a subset of serotype III GBS. Studies by Brady et al. (1996) suggested

that the δ and ε are markers for a more pathogenic subset of serotype III GBS, and may also play a role in pathogenicity with this subset. The two antigens were identified more frequently on isolates from septic vs. colonized sources, and expression of these antigens was associated with resistance to intracellular killing in an opsonophagocytosis assay (Brady et al. 1996) The major goals of the research project described in this dissertation were to isolate and characterize the δ and ε antigens, identify the genetic basis for antigen expression, and determine the association of antigen expression with pathogenicity. In addition to δ and ε, a cytosolic enzyme, glyceraldehyde-3-phosphate dehydrogenase was also identified as associated with the GBS surface and was partially characterized.

**Isolation and Characterization of the GBS δ and ε Antigens**

Serotype III GBS are a highly pathogenic serotype involved in all manifestations of GBS disease. In an attempt to identify and characterize surface components that may contribute to the virulence of GBS, Brady et al. (1988) identified an immunologic reactivity associated with a subset of serotype III GBS, designated δ. Adsorption of a rabbit antiserum made against a δ-expressing serotype III strain (J48) with a δ-negative serotype III strain (DL700) rendered this antiserum more highly specific for the δ antigen on GBS whole cells. When this antiserum was subsequently adsorbed with GBS strain A909 (serotype Ic/αβγδ) to remove antibodies against δ, antibody binding remained against all serotype III strains that express the δ antigen. This newly identified antigen was designated ε. Initial opsonophagocytosis assays and epidemiological data (Brady et al. 1996) suggested that expression of the δ and ε antigens are markers for a more pathogenic subset of serotype III GBS and may also be involved in pathogenicity. Therefore, experiments were conducted to isolate and characterize the δ and ε antigens.

In Chapter 2 it was shown that both the δ and ε antigens could be extracted from GBS whole cells by sonication. Adsorbed rabbit antisera were specific for δ and ε on whole bacterial cells, but also recognized unrelated antigens contained in cellular extracts. Therefore, a whole cell competition ELISA was used to detect δ and ε specifically in GBS extracts. Material extracted from strain J48 by sonication was able to inhibit binding of both anti-δ and anti-ε antisera to J48 whole cells, while similar material from strain DL700 was not. This indicated that sonication was an effective method to extract δ and ε from GBS whole cells.

Sonicated material from J48, in comparison with that from DL700, was subjected to anion-exchange chromatography in an attempt to isolate and identify δ and ε. Comparison of the $OD_{280}$ elution profiles of sonic extracts of J48 and DL700 showed no significant differences despite the presence of the δ and ε antigens in the J48 sample. These antigens were detected in column fractions by competition ELISA. Material from J48 eluted from the column with 0.2M NaCl inhibited anti-δε antiserum binding to A909 (serotype Ic/αβγδ) whole cells and anti-ε antiserum binding to J48 (serotype III/δε). This suggested that δ and ε eluted from the anion-exchange column in a peak with 0.2 M NaCl. These column fractions also contained material reactive with antibodies against the group B carbohydrate and serotype III capsular polysaccharide, suggesting that multiple surface components are associated and elute from the column as a complex with very low levels of NaCl. The possibility exists that sonication of GBS whole cells was not sufficient to disrupt cell wall complexes, causing multiple surface components to co-purify in the same column fractions. Increasing the time that the GBS whole cells were sonicated may have further disrupted complexes, but this was not tested.

To partially characterize the cell-associated δ and ε antigens, whole bacteria were treated with proteinase K or sodium metaperiodate and tested for reactivity with appropriate antiserum. The δ antigen on A909 whole cells was sensitive to treatment with proteinase K, but not to sodium metaperiodate, suggesting that the δ antigen is a protein. The ε antigen on J48 whole cells was completely destroyed by treatment with proteinase K and decreased by 50% after treatment with sodium metaperiodate, suggesting that both protein and carbohydrate components contribute to the ε antigen.

Western immunoblot analysis with appropriate antisera was used to identify novel immunoreactive bands associated with δ and ε surface expression. Adsorbed anti-δ specific antiserum derived from a polyclonal anti-A909 whole cell antiserum is difficult to work with due to its low titer. Therefore, an immunoprecipitation method was used in an attempt to affinity purify anti-δ antibodies from the higher titer anti-δε antiserum. This reagent, enriched for anti-δ antibodies, was used to identify immunoreactive bands unique to δ-positive strains in either anion-exchange column fractions or SDS extracts. Western immunoblot analysis identified an immunoreactive band at ~150 kDa present in an anion-exchange fraction from a J48, but not DL700 sonic extract. This protein was subjected to N-terminal sequencing and demonstrated homology (16/17 amino acids) with the deduced amino acid sequence of *gbs0332* (Glaser et al. 2002), an acyl-carrier protein (ACP) of GBS. Western immunoblot analysis of SDS-extracted material from multiple δ-surface positive and δ-surface negative GBS whole cells identified immunoreactive bands unique to these subsets. An immunoreactive band migrating at ~25 kDa present only in samples from δ-positive strains and a ~35-kDa immunoreactive band present only in samples from δ-negative strains were subjected to N-terminal

sequencing. Both bands demonstrated sequence homology with the deduced amino acid sequence of the same ACP of GBS as the ~150 kDa band isolated from the J48 sonic extract. These results were unexpected. One would not predict a common sequence would be identified for δ-positive and δ-negative strains. ACP is an enzyme known to carry substrates to six separate enzymes for sequential processing during fatty acid biosynthesis (Wakil et al. 1983). It is unknown why ACP sequence was obtained for bands migrating at ~150, 35, and 25 kDa when ACP itself is only 10 kDa. Anti-δ antibodies were not reactive with recombinant GBS ACP indicating that ACP does not represent the δ antigen itself. Recent studies by Gully et al. (2003) showed that ACP from *E. coli* can interact with proteins possibly by forming disulfide bonds. This would explain a size discrepancy, but it is still unknown why these bands are reactive with anti-δ antibodies from both δ-positive and δ-negative strains. ACP can interact with proteins in addition to those necessary for fatty acid biosynthesis (Gully et al. 2003), so it is possible that ACP might interact specifically with the δ antigen. In that case, immunoreactivity of a variant band with serotype III/δ-surface negative strains could be explained if the form of the antigen associated with these strains is masked in the context of the whole cell. It is also possible that association of ACP with δ is not specific, but the difference in ACP migration is a marker for δ-positive and δ-negative strains. Additional experimentation beyond the scope of this project is necessary to fully explore the relationship between δ expression and ACP.

Western immunoblot of an ε-containing anion-exchange column fraction was used to identify ε as a high molecular weight antigen (>250 kDa) that was not stainable by conventional protein staining techniques. Attempts to obtain N-terminal sequence

information from this material were unsuccessful. Because ε was localized to >250 kDa by Western immunoblot, material presumed to contain ε was eluted from SDS-polyacrylamide gels at this apparent molecular mass. Gel-purified material from strain J48 (serotype III/δε), but not DL700 (serotype III), was reactive with anti-ε antiserum. Neither eluted material was reactive with anti-δ or anti-serotype III antiserum. Eluted material from both strains was reactive with a group B carbohydrate typing reagent suggesting a relationship with this group specific antigen. However, only two of the component group B carbohydrate sugars, rhamnose and galactose were detected in preparations from both strains.

Preliminary studies with proteinase K and sodium metaperiodate treated J48 whole cells suggested that ε was comprised of both protein and carbohydrate components. Epsilon-containing material from J48, and corresponding material from DL700, were subjected to both carbohydrate and amino acid analysis. Carbohydrate analysis of eluted material from both strains indicated the presence of rhamnose, mannose, galactose, and glucose. The group B carbohydrate consists of rhamnose, galactose, *N*-acetylglucosamine, and glucitol (Michon et al. 1988), supporting the finding of anti-group B reactive material in the gel-eluted preparations from both strains. This would indicate that the presence of the entire native group antigen is not necessary for reactivity with anti-group B antibodies. Interestingly, the eluted material from each strain also contained mannose and glucose, although the linkages of these sugars to other sugars or a protein are unknown. It is possible that differences in linkages of the same sugars could account for ε antigenic reactivity compared to δε-negative strains. Amino acid analysis of the eluted material from J48 and DL700 showed amino acids in similar ratios with no

obvious differences upon comparison of the two strains. Amino acid analysis suggested the presence of a protein rich in glycine or serine (~30% of total amino acids) in both samples.

Although the exact nature of the ε antigen remained unknown, an attempt was made to generate a more specific antiserum against ε. Epsilon-containing material present in a sonic extract from J48 (serotype III/δε) and corresponding material from DL700 (serotype III) was gel-purified from multiple SDS-polyacrylamide gels. These preparations were used as immunogens in rabbits. Comparison of the reactivity of the resulting antiserum was used to identify ε in extracts of GBS by Western immunoblot. The antiserum made against the J48 material recognized a >250-kDa antigen present in all ε-positive, but not ε-negative strains. The antiserum made against the DL700 material did not recognize bands unique to ε-negative strains. These antisera were utilized in subsequent passive protection experiments using a murine model of neonatal sepsis to evaluate the potential role of anti-ε antibodies against lethal GBS challenge.

**Determination of Genetic Components Involved in δ and ε Expression**

Initial work to isolate and characterize the δ antigen suggested that δ was associated with one of the three acyl-carrier proteins (ACP) identified in the GBS genomic sequence (Glaser et al. 2002). Therefore this GBS *acp* was cloned and the recombinant ACP expressed in *E. coli*. Anti-δ antiserum did not bind to the recombinant ACP. Therefore, although altered migration of ACP is associated with δ expression, ACP does not represent the unique δ antigen detectable on the cell surface of a subset of serotype III GBS. The specific ACP identified by N-terminal sequencing is located within an operon containing genes for fatty acid biosynthesis. It is unknown what, if any,

role this ACP plays in δ-surface expression. One experimental approach to address this question would be to immunize rabbits with a synthetic peptide with sequence unique to this ACP. Antiserum generated against the peptide could be used in immunoprecipitation experiments to capture and identify proteins interacting with ACP. Mutants could then be made to eliminate expression of candidate proteins and evaluate δ expression. Conversely, candidate proteins could be subcloned into an expression vector and assayed directly for anti-δ binding. It is possible that the altered migration of ACP associated with differences in immunoreactivity of δ-positive and δ-negative strains seen in Chapter 2 is artifactual and does not reflect a specific association with variant forms of δ in these two subsets of strains. Additional experiments beyond the scope of this project are necessary to elucidate the possible role of GBS ACP in δ-expression.

Amino acid analysis of high molecular weight ε-containing material from J48 and the corresponding material from DL700 suggested the presence of a protein rich in either glycine or serine. One open reading frame (ORF) within the genomic sequence of a serotype III strain (Glaser et al. 2002) was identified as having a deduced amino acid sequence with a high percentage of serine (~32% of total). PCR amplification of this ORF was attempted. Amplification was only successful for GBS strains that did not express ε. To test whether DNA encoding a serine-rich repeat (Srr) protein from ε-positive strains was unable to be amplified due to the absence of a gene encoding this protein, Southern hybridization was employed. The PCR product from DL700 (serotype III) was allowed to hybridize with digested chromosomal DNA from a panel of GBS strains. The PCR product hybridized with all ε-negative strains, but not ε-positive strains, confirming the results observed by PCR amplification. The combination of PCR

amplification and DNA hybridization suggested that the Srr-encoding ORF from the GBS genomic sequence database (Glaser et al. 2002) was not present in the chromosome of ε-negative strains.

Although GBS are not known to contain plasmids or phage, the possibility existed that Srr might be encoded by a heretofore unrecognized extra-chromosomal element. To screen for *srr* mRNA, the labeled PCR product from DL700 was allowed to hybridize with total RNA extracted from an ε-positive and an ε-negative strain. The probe only hybridized with RNA from the ε-negative strain demonstrating that *srr* was not contained on an extra-chromosomal element in ε-positive strains. The inverse relationship between δε-expression and the presence of the *srr* ORF suggested that the NEM316 GBS sequenced strain would be δε-negative. A whole-cell ELISA demonstrated this result.

The *srr* ORF from the sequenced GBS genome (Glaser et al. 2002) is located in a locus with significant similarity to a locus in *Streptococcus gordonii* described by Bensing and Sullam (2002), with accessory *secA* and *secY* homologues specific for the export of the serine-rich repeat protein GspB. Since the *srr* ORF was absent in ε-positive GBS, PCR amplification was used to determine if additional ORFs within this locus might also be absent in ε-positive GBS. Of the eleven ORFs tested, ε-positive strain J48 (serotype III/δε) only contained two, *mutT* and *uvrB*. These genes are located on the 5' and 3' ends of the *secA2-Y2* operon. The nine other genes tested were only amplified from DL700, an ε-negative strain. Hence, in addition to lacking the *srr* ORF itself, δε-positive serotype III GBS appear to lack a 24-kb segment of DNA present between *mutT* and *uvrB* in δε-negative strains. The basis for additional antigenic reactivities associated with a lack of genes within the *secA2-Y2* locus is currently unknown.

Work by Takahashi et al. (1998) identified an apparently more pathogenic subset of serotype III GBS based on restriction digest patterns (RDP) of chromosomal DNA, and Brady et al. (1996) suggested that δ/ε expression was a marker for a more pathogenic subset of serotype III GBS based on epidemiological and opsonophagocytosis data. The δ antigen is expressed by strain A909 (Ic/αβγδ) but to date ε has been found exclusively on serotype III/δ-positive strains. To determine if ε expression was correlated specifically with a particular RDP III lineage, GBS strains of RDP types III-1, 2, 3, and 4 were assayed for ε expression. All RDP III-3 strains were ε-positive, while all non III-3 strains were ε-negative These results suggest RDP III-3 and δε-positive serotype III GBS represent the same genetic lineage.

Bohnsack et al. (2002) used genomic subtractive hybridization to identify DNA sequences unique to RDP III-3 strains. One clone, designated DY-3, contained DNA encoding a large putative protein rich in serine, similar but not identical to the Srr protein from the genomic sequence of NEM316 (Glaser et al. 2002). PCR analysis and DNA hybridization confirmed the presence of a variant *srr* ORF in chromosomal DNA from δε-positive strains. These data were consistent with amino acid analysis of ε-containing material from both J48 (serotype III/δε), and corresponding material from DL700 (serotype III), which suggested the presence of a serine-rich protein in both samples.

The *srr* ORF from J48 (serotype III/δε) was cloned and the recombinant Srr protein was expressed in *E. coli*. Anti-ε antibodies bound to the recombinant protein, suggesting this Srr represents the protein backbone of the putative ε glycoprotein. Surface-localized ε antigen on J48 was sensitive to proteolysis with proteinase K. While sodium metaperiodate sensitivity also suggests the contribution of carbohydrate epitopes to anti-ε

reactivity, the exact contribution of carbohydrate moieties to ε are as yet unknown. The similar carbohydrate composition of δε-positive and δε-negative material isolated from strains J48 and DL700 suggest that anti-ε antibodies may recognize a difference in linkages between sugars rather than a difference in the sugars themselves. It is possible, but unlikely, that sensitivity of the ε antigen to treatment with sodium metaperiodate is a result of modification of amino acid residues. The presence of sugars in the serine-rich, gel-purified material reactive with anti-ε antibodies and reports of extensive glycosylation of serine-rich proteins recently identified in other streptococci suggest that carbohydrate modification does contribute to ε reactivity. Stephenson et al. (2002) have reported that the Srr protein from *Streptococcus parasanguis* (Fap1) is extensively modified by sugar moieties. The Srr protein from J48 is predicted to migrate at ~125 kDa, yet ε reactivity is detected at >250 kDa, suggesting post-translational modification. Carbohydrate composition analysis of ε-containing material from J48 demonstrated the presence of four sugars including two in common with the group B antigen. The relationship of ε and Srr to the group antigen is unknown, but the reactivity of the isolated material with the group typing reagent is intriguing and suggests a close association.

A comparison of the genomic sequence of strain NEM316 (serotype III, RDP III-1) (Glaser et al. 2002) and the partial DY-3 sequence (Bohnsack et al. 2002) shows significant differences in the DNA flanking the variant *srr* ORFs. A *secY2* homologue is located only 113 nt downstream of *srr* in the DY-3 clone derived from the RDP III-3, δε-positive strain 874391. The amount of DNA between *srr* and *secY2* in NEM316 is 12 kb (Glaser et al. 2002). This 12-kb segment contains six ORFs with homology to

glycosyltransferases. The role of these putative glycosyltransferases is unknown. The DY-3 clone includes DNA only through approximately half of the *secY2* homologue. It is likely that ε-positive strains also contain as yet unidentified glycosyltransferases involved in modification of their variant Srr since it is probable that both Srr variants are glycosylated.

**The Association of δ and ε Expression with Serotype III GBS Pathogenesis**

Initial experiments with J48 (serotype III/δε) and DL700 (serotype III) revealed that J48 had a $LD_{90}$ approximately 4 logs lower than that determined for DL700. Although the pathogenicity of GBS strains in a neonatal mouse model varies from strain to strain, a 4-log difference in lethal dose is extreme. Experiments with multiple δε-positive and δε-negative strains confirmed a difference in $LD_{90}$ on the same order of magnitude suggesting that this difference is indeed associated with δ/ε expression and not a result of strain-strain variation between J48 and DL700.

The ability to protect neonatal mouse pups against lethal challenge after administering mothers antiserum made against GBS antigens other than the capsular polysaccharide has been demonstrated (Brodeur et al. 2000, Cheng et al. 2001, 2002, Gravekamp et al. 1999, Larsson et al. 1996, Madoff et al. 1991, 1992, 1994, Stalhammar-Carlemalm et al. 1993). Passive protection assays using polyclonal rabbit antiserum made against the ε-containing material from J48 (serotype III/δε), and the corresponding material from DL700 (serotype III), offered insight into the protective ability of anti-ε antibodies in a neonatal mouse model. Antiserum made against the semi-purified ε antigen (also containing material reactive with group B specific antibodies) did not protect neonates from lethal GBS challenge. Antiserum made against the ε-negative

material derived from DL700 was also non-protective. These results suggest that ε is not the target of protective immunity. This does not mean, however, that ε might not play a role in GBS pathogenesis. In both *S. gordonii* and *S. parasanguis*, the respective Srr proteins mediate attachment to either platelets or salivary-coated hydroxyapatite (Bensing and Sullam 2002, Wu et al. 1998, Wu et al. 1999). Hsa from *S. gordonii* has also been shown to bind to sialic acid (Takahashi et al. 2002). It is unknown what role the GBS Srr may play in pathogenesis; however, an isogenic mutant lacking *srr* would likely provide information. Attempts to generate such a mutant have been unsuccessful to date, largely because of the difficulty in transforming a highly encapsulated organism that is not naturally competent.

A neonatal mouse model is used extensively in lethal dose and passive protection experiments in GBS (Brodeur et al. 2000, Madoff et al. 1991, 1992, Paoletti et al. 1994, Rodewald et al. 1992, Stalhammar-Carlemalm et al. 1993). However, there are drawbacks to this approach. This method calls for intraperitoneal injection of GBS, bypassing two important aspects of GBS pathogenesis, adhesion and invasion. If the Srr of GBS has an adhesive function similar to that described for other Srr proteins, the loss of Srr in an isogenic mutant may not show a difference in pathogenicity in this model. Even if differences are observed, it is possible that the protein could have multiple functions in GBS pathogenesis or a function unrelated to initial colonization. Since serotype III GBS are most often isolated from cases of meningitis (Dillon et al. 1987, Wenger et al. 1990), and since serotype III GBS are better at invading brain microvascular cells than other serotypes (Nizet et al. 1997), it would be interesting to know if brain cell invasion assays could be taken a step further to compare ε-positive to

ε-negative serotype III GBS. Likewise, it would be interesting to compare wild-type and *srr*-deficient mutants to determine if Srr from δε-positive and/or δε-negative strains offers the organisms a selective advantage in invasion of brain microvascular tissue and resulting meningitis.

**Characterization of Group B Streptococcal Glyceraldehyde-3-Phosphate Dehydrogenase: Surface Localization, Enzymatic Activity, and Protein-Protein Interactions**

Cytosolic proteins can be associated with the cell surface of GBS, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Hughes et al. 2002, Pancholi and Fischetti 1992, Seifert et al. 2003). An anion-exchange column fraction of a sonic extract of GBS strain J48 (serotype III/δε) that contained group-specific reactive antigen, capsular polysaccharide, and the δ and ε antigens also contained a high molecular weight protein that was homologous to group A streptococcal GAPDH (Plr). An anti-Plr monoclonal antibody bound to GBS whole cells suggesting GAPDH surface localization. The anti-Plr monoclonal antibody also bound to a ~173.5-kDa protein by Western immunoblot, presumably the tetrameric form of GAPDH. The observed migration of GBS GAPDH is larger than that reported for GAS GAPDH (Pancholi and Fischetti 1992). As mentioned before, it is unknown if sonication completely disrupts cell wall complexes. It is possible that GAPDH is complexed with other surface structures, and thus the migration of tetrameric GAPDH may be altered. It is also possible that GAPDH is post-translationally modified (Fernandes et al. 1992), but studies to determine potential modifications have not yet been performed.

Southern analysis, as well as information from published genomic sequence databases (Glaser et al. 2002, Tettelin et al. 2002) indicated that GBS possess a single

gene that encodes both cytosolic and surface-localized GAPDH. Although unlikely, it is possible that a second, highly divergent *gapC* gene may be present on the GBS chromosome. Other microorganisms have been reported to contain multiple GAPDH genes *Saccharomyces cerevisiae* has three GAPDH genes (Holland et al. 1983) and *E. coli* has two (Alefounder and Perham 1989).

The ability of streptococci to bind to cytoskeletal and extracellular matrix proteins has been postulated to contribute to pathogenicity (Boyle et al. 1997, D'Costa and Boyle 2000, Pancholi and Fischetti 1992, Winram and Lottenberg 1996). The ability of GBS GAPDH to bind to multiple proteins was assayed. Lys-plasminogen, glu-plasminogen, actin, and fibrinogen were able to interact with GAPDH depending on which moiety was immobilized. The binding of host proteins by GAPDH can vary from strain to strain (D'Costa and Boyle 2000). Since J48 was the only GBS strain tested, it is possible that different binding profiles would be observed if additional strains were tested as well. Further experiments are required to determine if any of the protein-binding interactions contribute to adhesion and (or) invasion during GBS infections and if GAPDH is a factor in GBS pathogenesis.

## Conclusions

The purpose of this project was to identify components responsible for δ and ε antigenic reactivity and to characterize these antigens. Three specific aims regarding the δ and ε antigens and characterization of another surface protein, glyceraldehyde-3-phosphate dehydrogenase were accomplished in the work presented in this dissertation. The specific aims accomplished included isolation and partial characterization of the δ and ε antigens; evaluation of the genetic basis of δ and ε expression; and evaluation of

the association of δ and ε expression with the pathogenesis of serotype III GBS. It was found that the δ antigen is most likely a protein. It is associated with a GBS ACP, but whether this relationship is specifically related to δ immunoreactivity remains to be seen. The ε antigen is comprised of both protein and carbohydrate components, most likely similar to GspB and Hsa of *S. gordonii* and Fap1 of *S. parasanguis* (Bensing and Sullam 2002, Takahashi et al. 2002, Wu et al. 1999). These large glycoproteins are comprised extensively of a repetitive di-serine (XS) motif and are reported to serve as adhesins for different substrates.

Serotype III/δε-positive GBS were shown to correspond to the more pathogenic RDP III-3 subset of serotype III strains based on restriction digest patterns. Anti-ε antibodies bind to recombinant Srr from J48, suggesting that recombinant Srr represents the protein backbone of the ε antigen. RDP III/δε-positive strains have a Srr protein different than that from non-III-3 strains. The variant *srr* genes are contained within variant loci containing accessory *sec* gene homologues as well. The information obtained to date regarding the organization of these variant loci is summarized schematically in Figure 3-8. Interestingly, based on the sequence of the DY-3 clone reported by Bohnsack et al. (2002), the *srr*-containing locus associated with RDP III-3/δε-positive strains appears similar to that recently reported for *Staphylococcus aureus* and *Staphylococcus epidermidis* in that the *secY2* homologue lies immediately downstream of the *srr* gene itself (Takamatsu et al. 2004). BLAST analysis (Altschul et al. 1997) was performed and demonstrated that the primary sequence homologies of the *srr* and *secY2* genes from the RDP III-3/δε-positive variant are higher with the staphylococcal genes than with those from non-RDP III-3/δε-negative GBS (data not shown). In light of the presence of

insertion sequences in the vicinity of the *srr*-containing locus in RDP III-3/δε-positive GBS, it is tempting to speculate that a horizontal gene transfer event from staphylococcus is associated with increased pathogenesis of this lineage.

Although the role of δ and ε in pathogenesis is not completely understood, $LD_{90}$ studies indicate that the presence of δ and ε is at the very least, a marker for a more pathogenic lineage of serotype III GBS. Antibodies to ε were not protective in a neonatal mouse model, indicating that ε would likely not represent an effective vaccine candidate. However, the clinical utility of δ and ε as important diagnostic indicators predictive of high-risk strains is strongly suggested. Further work with an isogenic *srr* mutant or complementation with *srr* variants will be necessary to identify a direct role, if any, of Srr expression in GBS pathogenesis.

In addition to δ and ε, GAPDH was identified as a surface-localized protein present on all serotypes of GBS tested. GBS GAPDH was enzymatically active and able to interact with a number of cytoskeletal and extracellular matrix proteins, which may contribute the adhesive ability of GBS.

The work presented in this study contributes to an increased understanding of the antigenicity and genetics of a highly pathogenic lineage of serotype III GBS

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## BIOGRAPHICAL SKETCH

Kyle Nikoli Seifert was born on May 11, 1975 in Wells, Minnesota. He graduated from Fairmont Senior High School in 1993, and began his undergraduate studies at Augustana College in Sioux Falls, South Dakota that same year. At Augustana College, he was an offensive lineman for the varsity football team. In 1998, Kyle received a Bachelor of Arts degree in biology. After working for 6 months for a mail-order pharmaceutical company after graduation, he moved to Gainesville, Florida, and entered the Interdisciplinary Program in Biomedical Sciences at the University of Florida. During this time, he specialized in microbiology and immunology in the Department of Oral Biology. On December 18$^{th}$, 1998, Kyle married Rochelle Schmit; and on October 1$^{st}$, 1999 Rochelle gave birth to the couple's first child, Allison Nicole Seifert. Kyle plans on joining the Department of Biology at James Madison University as an Assistant Professor, where he will teach microbiology to undergraduate students.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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